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Non-instructional roles for Notch signaling in T cell development

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Non-instructional roles for Notch signaling in T cell development

Abstract

Mammalian immunity requires the presence of a broad and diverse repertoire of antigen receptors that can recognize the virtually infinite number of pathogenic epitopes encountered over a lifetime of a host, along with the ability to flexibly mount organized and pathogen-specific immune responses, as orchestrated by cytokines and CD4+ helper T (Th) cells. The Notch signaling pathway plays a critical role both in the generation of the T cell repertoire and in Th cell differentiation. However, while Notch is well understood to instruct early T lineage development at the expense of alternate lineages, it is unclear how Notch regulates the differentiation of a common naïve progenitor into one of the many Th cell subsets. To clarify the molecular mechanism used by Notch to influence Th cell differentiation, the dynamics of Notch target binding and gene regulation were analyzed at early time points after T cell activation. Rather than finding that Notch signaling acts via the canonical instructional paradigm, these studies find that Notch acts as an unbiased integrator of environmental differentiation cues, such that it simultaneously promotes the differentiation of multiple inflammatory Th cell populations. These findings are supported by in vivo gain-of-function studies in which Notch signaling is constitutively activated in peripheral T cells. Unlike previous work showing that hyperactivation of the Notch pathway in T lineage progenitors yields aggressive T cell acute lymphoblastic leukemia, these mice go on to develop a lethal autoinflammatory disorder, resulting from the Notch pathway promoting the differentiation of Th1, Th2, and Th17 cells. Additionally, these studies reveal that Notch signaling acts to destabilize regulatory T cell differentiation. Altogether, the work presented in this thesis evinces a novel non-instructional paradigm for Notch signaling, with broad implications for our understanding of Th cell differentiation, hematopoietic development, and cancer biology.

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NON-INSTRUMENTAL ROLES FOR NOTCH SIGNALING IN T CELL DEVELOPMENT

Will Harrison Bailis

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ABSTRACT

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Will Harrison Bailis

Warren S. Pear

Mammalian immunity requires the presence of a broad and diverse repertoire of antigen receptors that can recognize the virtually infinite number of pathogenic epitopes encountered over a lifetime of a host, along with the ability to flexibly mount organized and pathogen-specific immune responses, as orchestrated by cytokines and CD4⁺ helper T (Th) cells. The Notch signaling pathway plays a critical role both in the generation of the T cell repertoire and in Th cell differentiation. However, while Notch is well understood to instruct early T lineage development at the expense of alternate lineages, it is unclear how Notch regulates the differentiation of a common naïve progenitor into one of the many Th cell subsets. To clarify the molecular mechanism used by Notch to influence Th cell differentiation, the dynamics of Notch target binding and gene regulation were analyzed at early time points after T cell activation. Rather than finding that Notch signaling acts *via* the canonical instructional paradigm, these studies find that Notch acts as an unbiased integrator of environmental differentiation cues, such that it simultaneously promotes the differentiation of multiple inflammatory Th cell populations. These findings are supported by *in vivo* gain-of-function studies in which Notch signaling is constitutively activated in peripheral T cells. Unlike previous work showing that hyper-activation of the Notch pathway in T lineage progenitors yields aggressive T cell acute lymphoblastic leukemia, these mice go on to develop a lethal autoinflammatory disorder, resulting from the Notch pathway promoting the

differentiation of Th1, Th2, and Th17 cells. Additionally, these studies reveal that Notch signaling acts to destabilize regulatory T cell differentiation. Altogether, the work presented in this thesis evinces a novel non-instructional paradigm for Notch signaling, with broad implications for our understanding of Th cell differentiation, hematopoietic development, and cancer biology.

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LIST OF PUBLICATIONS

The content of this thesis contains portions of modified text and figures from the following published articles, as well as additional work currently in preparation for submission:

Bailis, W*, Yashiro-Ohtani Y*, Fang TC, Hatton RD, Weaver CT, Artis D, Pear WS. (2013). Notch simultaneously orchestrates multiple helper T cell programs independently of cytokine signals. *Immunity*.

Bailis W, Pear WS. “The Molecular Basis of T Cell Development and How Epigenetic/Transcriptional Deregulation Leads to T-ALL.” (2014). In Bonifer, C and Cockerill, PN (Eds.), *Transcriptional and Epigenetic Mechanisms Regulating Normal and Aberrant Blood Cell Development*.

ATTRIBUTIONS

All the ideas and illustrations presented within this thesis are those of Will Bailis. The data in Figure 2-1 were generated by David Artis in collaboration with Terry Fang. All other experiments were performed by Will Bailis in collaboration with Yumi Ohtani.

CHAPTER 1: Introduction

The mammalian immune system is faced with the daunting challenge of generating a T cell receptor (TCR) repertoire large enough to cover every potential antigen a host will encounter in their lifetime from a limited number of progenitors. To accomplish this task, the few progenitors that enter the thymus must expand by several orders of magnitude in order to generate sufficient genetic material to survive antigen receptor recombination and TCR selection. Even after this is accomplished and a mature T cell enters the periphery, it will need to further differentiate to acquire its effector function. As cognate antigen recognition alone is not sufficient to clear a pathogen, adaptive immune cells must orchestrate a persistent and coherent response from both the adaptive and innate arms of the immune system, a task that requires the differentiation of CD4⁺ helper T (Th) cells into one of several subsets. Notch signaling is critical for both of these development processes, yet given the disparate demands of early and late T lineage development it is unclear how such a basic signaling module can organize two distinct differentiation mechanisms in the same lineage.

While Notch signaling is understood to instruct early T lineage development, there is little consensus as to how the Notch pathway influences Th cell differentiation (Radtke et al., 2013). Pioneering work with *in vitro* Th cell cultures yielded a paradigm in which the quality and fidelity of Th cell differentiation was governed by a linear model: a lineage inducing cytokine initiates a transcriptional program governed by a lineage defining transcription factor, which in turn licenses that cell to produce a lineage defining cytokine (O'Shea and Paul, 2010). While this highly stereotyped progression holds well in traditional *in vitro* differentiation systems, activated CD4⁺ T cells are often exposed to a

complex milieu of differentiating factors *in vivo*, each acting to push a Th cell down a different lineage path. Within this framework, the need for molecules that function to maintain signal within this noisy sea of differentiating cues becomes apparent. This thesis contends that the Notch pathway functions as one such signal-integrating module in the peripheral CD4⁺ compartment, distinct from its classical instructional role in early T lineage development. The findings presented suggest that Notch signaling exerts distinct mechanistic functions in early and late T lineage development and evinces a novel signal integration model for Notch in hematopoiesis.

Overview of $\alpha\beta$ -thymocyte development

Although many hematopoietic progenitor cells have the capacity to become T cells, only a few migrate into the thymus (Bhandoola et al., 2007; Ng et al., 2009). Upon entry, these thymic emigrants respond to their new environment by proliferating extensively and initiating the T cell developmental program (Figure 1-1). The engagement of the T cell lineage program involves the inactivation of stem cell and progenitor genes, the activation of lineage specific factors, and the restriction of alternate fates (Rothenberg, 2012). Intimately intertwined with the transcriptional reprogramming of thymocytes, the ongoing process of TCR rearrangement provides an additional level of regulatory complexity to T cell development and can begin even before progenitors enter the thymus (Allman et al., 2003; Igarashi et al., 2002).

In this manner, T cell development can be divided into multiple stages by the initiation of the T cell transcriptional program and then several developmental checkpoints governed by the ordered rearrangement of the TCR and the selection of a thymocyte's antigen

receptor against self-peptides (Figure 1-1). These developmental stages are defined by a set of surface markers that correlate with T cell maturation. The earliest populations of thymocytes are defined by their lack of expression of the co-receptors CD4 and CD8, and thus termed double negative (DN) cells. DN cells can be further subfractionated based upon their expression of CD25, the IL-2 receptor α -chain, and CD44, a cell adhesion receptor. The first cells to enter the thymus, DN1, are defined as CD25⁻CD44⁺ and progress to CD25⁺CD44⁺ DN2 cells, CD25⁺CD44⁻ DN3 cells, and then CD25⁻CD44⁻ DN4 cells. From DN1 through DN3, thymocytes rearrange the TCR β , in a recombination activating gene (RAG) 1 and 2 dependent manner, and are held at a DN3 checkpoint that ensures functional TCR β expression, termed β -selection. Subsequent to the DN stages of T cell development, thymocytes become double positive (DP) cells, characterized by the surface expression of CD4 and CD8. At the DP stage, thymocytes test a fully rearranged TCR against self-peptides presented on the major histocompatibility complex (MHC), such that all thymocytes are capable of recognizing self-MHC (positive selection), but do not activate upon engagement of a self-peptide:MHC complex (negative selection). T cell development in the thymus ends when thymocytes successfully undergo selection and become single positive (SP) for either CD4 or CD8, as determined by the class of MHC that a thymocyte recognizes (MHC-I for CD8⁺ SP cells and MHC-II for CD4⁺ SP cells).

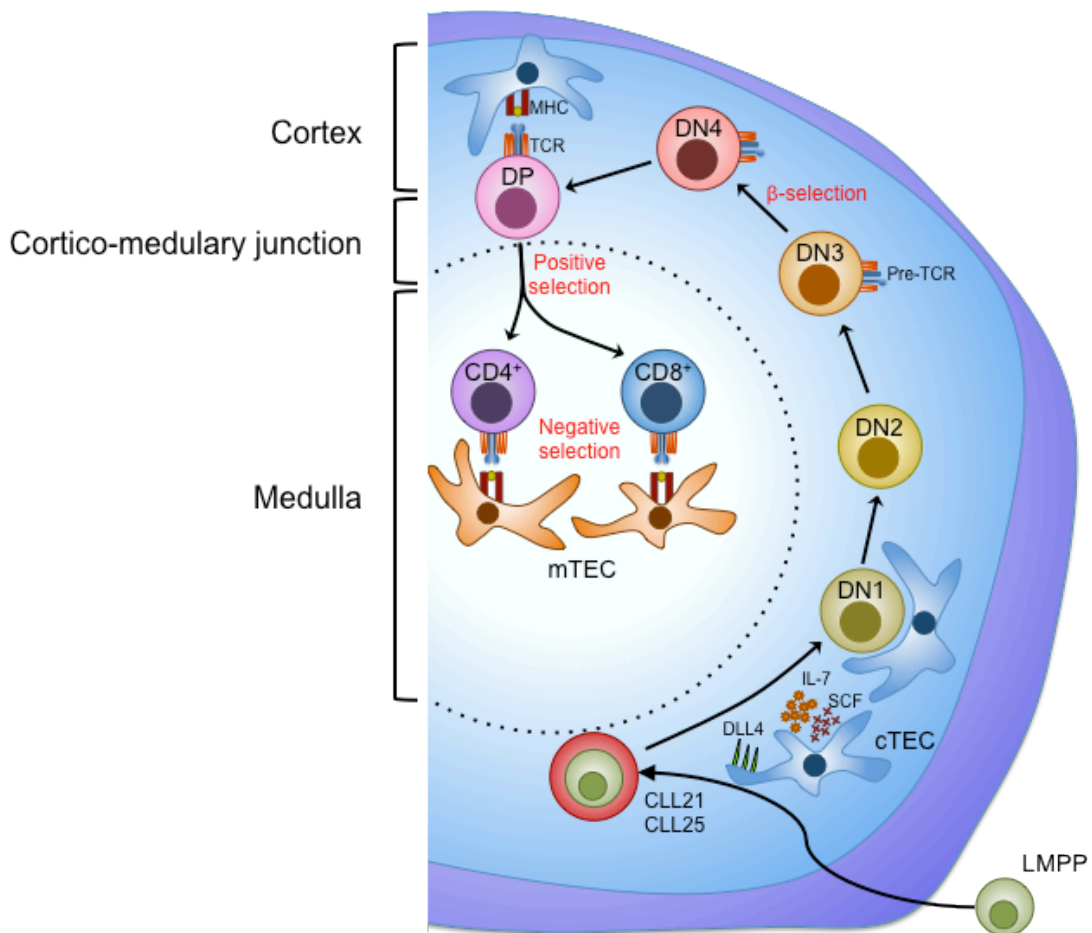


Figure 1-1: Stages of thymocyte development. Multipotent precursors are recruited to the thymus in a CLL21 and CLL25 dependent manner and are initially maintained by IL-7 and SCF survival signals, supplied by cortical thymic epithelial cells (cTECs). These early precursors, DN1 cells, then initiate the T-lineage program upon Notch ligand engagement and progress to the DN2 stage. At the DN3 stage, thymocytes begin to express the pre-TCR complex, composed of pre-TCR α and TCR β , and are licensed undergo β -selection and proceed to the DN4 stage. Once the TCR α locus is functionally rearranged and a mature TCR complex is expressed on the cell surface, DP thymocytes are subjected to positive selection by interacting with MHC bearing cTECs at the cortico-medullary junction. DP thymocytes then progress to either the CD4⁺ SP or CD8⁺ SP stage, depending on the class of MHC their TCR recognizes. Thymocyte development is completed after self-reactive SP thymocytes are eliminated by negative selection through their recognitions of self-peptides presented by medullary thymic epithelial

Building the T lineage identity

Multiple progenitor populations have been identified that can develop into T cells. However, the resident bone marrow lymphoid primed multipotent progenitors (LMPP) are understood to be the primary source of T cell precursors. Though they are enriched for T cell potential, LMPP's can also give rise to myeloid cells, NK cells, and B cells, but not erythrocytes or megakaryocytes (Yang et al., 2010). It is not until an LMPP expresses the chemokine receptors CCR7 and CCR9 that they are able to migrate to the thymus and begin T cell differentiation (Zlotoff et al., 2010).

Upon entry, the thymic epithelium provides these recent thymic emigrants (DN1 cells) with a potent milieu of both soluble factors, such as interleukin 7 (IL-7) and stem cell factor, as well as membrane bound ligands, the most critical of which are the Notch ligands delta-like ligand 1 (Dll1) and Dll4, that support T cell development, proliferation, and survival. Until a thymocyte successfully rearranges the TCR β -chain, these epithelial derived signals sustain the T cell program during the earliest stages of development, DN1 through DN3. Though the thymic environment strongly favors $\alpha\beta$ T cell development, other lineages can emerge from these pro-T cell populations and are supported within the thymus. The potential to differentiate into these alternate lineages is strongest at the DN1 stage and is almost completely lost by the DN3 stage (Rothenberg et al., 2010).

This loss of multipotentiality is mirrored by a progressive termination of a stem cell-like transcriptional identity and the emergence of a T cell specific transcriptional program, composed of Notch1, TCF1, GATA3, and Bcl11b (Rothenberg, 2012). Global mapping of histones and RNA-seq analysis of developing thymocytes has revealed that this

progressive loss of stem/progenitor-specific gene expression results from a variety of histone transformations at multiple time points during development (David-Fung et al., 2009; Tydell et al., 2007; Zhang et al., 2012). These data suggest that multiple biochemically and temporally distinct mechanisms govern the transition from a stem-like program to a T cell program, rather than a single switch (Zhang et al., 2012). Although much of the stem cell gene expression program is turned off, it is important to note that some genes sustain their expression throughout later stages of T cell development, such as *Myb* and *Ikaros*. While these sustained genes may play important regulatory roles, it has been suggested that they are unlikely to be rate-limiting factors for commitment (Rothenberg, 2012).

Notch signaling

Notch receptors are a family of evolutionarily conserved type-I transmembrane glycoproteins that play a critical role in large array of developmental processes, including multiple stages of T cell development (Bailis et al., 2013; Fang et al., 2007; Han et al., 2002; Maillard et al., 2004; Radtke et al., 1999). Mammals possess four different Notch receptors (Notch1-4) that share homology with *Drosophila* Notch, and five Notch ligands. The mammalian ligands Delta-like 1, 3, and 4 (Dll1, 3, 4) are homologous to the *Drosophila* Delta, while Jagged1 and 2 are structurally similar to the *Drosophila* Serrate. The extracellular portion of Notch is responsible for ligand interaction and is composed of epidermal growth factor (EGF-like) repeats, LIN12 repeats (LNRs), and a membrane-proximal heterodimerization domain (HD). In the absence of ligand, the LNR and the HD domains constitute a negative regulatory region (NRR) that ensures Notch receptors

remain membrane-bound and transcriptionally inactive. In response to ligand, the Notch intracellular domain (ICN) is released by proteolytic cleavage by gamma secretase and directly mediates signal transduction. ICN consists of a RAM domain, 7 ankyrin-like repeats (ANK), nuclear localization signal sequences, and a C-terminal PEST domain that regulates protein stability (Figure 1-2).

While there are structural differences amongst the four Notch receptors, all are activated in a ligand-dependent manner and converge on the same core transcriptional complex. Upon Notch receptor-ligand interaction between two neighboring cells, a series of proteolytic cleavage sites become exposed that lead to cleavage of an extracellular site by ADAM family metalloproteases and subsequent cleavage of a transmembrane site by a gamma-secretase complex. This proteolytic processing frees ICN from the membrane, which then translocates to the nucleus and complexes with the transcription factor RBPJ and MAML. MAML then recruits co-activators, such as p300 and PCAF, to form a large multiprotein transcriptional activation complex. In this manner, Notch signaling results from the conversion of a transmembrane receptor into a transcriptional activator, allowing for rapid changes in target gene expression (Figure 1-3).

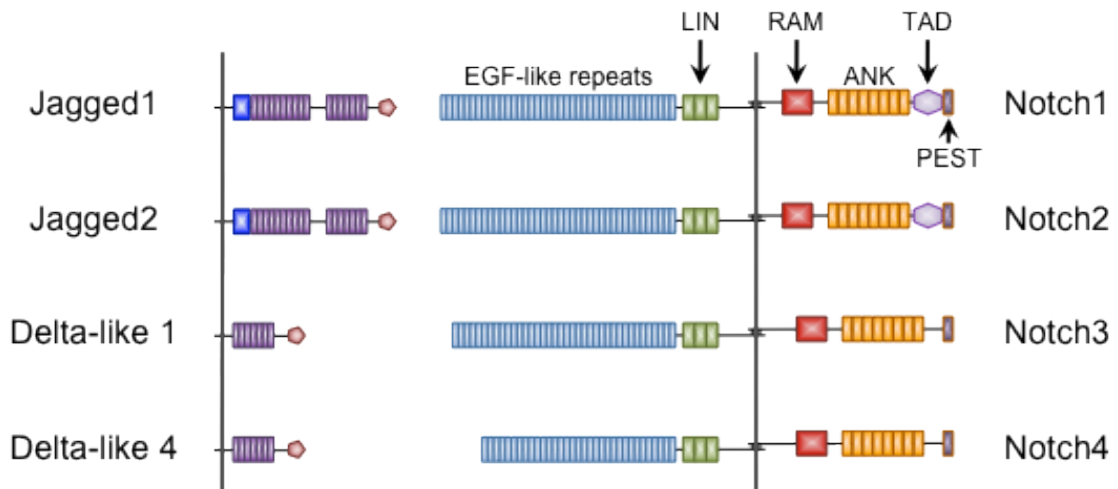


Figure 1-2: Structure of the mammalian Notch receptors and ligands. There are four mammalian Notch receptors, Notch1-4, each of which can be activated by one of four activating Notch ligands: Jagged1, Jagged2, Delta-like 1, and Delta-like 4. All four Notch receptors contain an extracellular domain composed of a series of EGF-like repeats followed by a LIN repeat containing region. The intracellular portion of the Notch receptors consists of a RAM domain, a series of ankyrin-like repeats (ANK), and a PEST domain that regulates protein stability. Notch1 and Notch2 additionally contain a intracellular transcriptional activation domain (TAD), unlike Notch3 or Notch4.

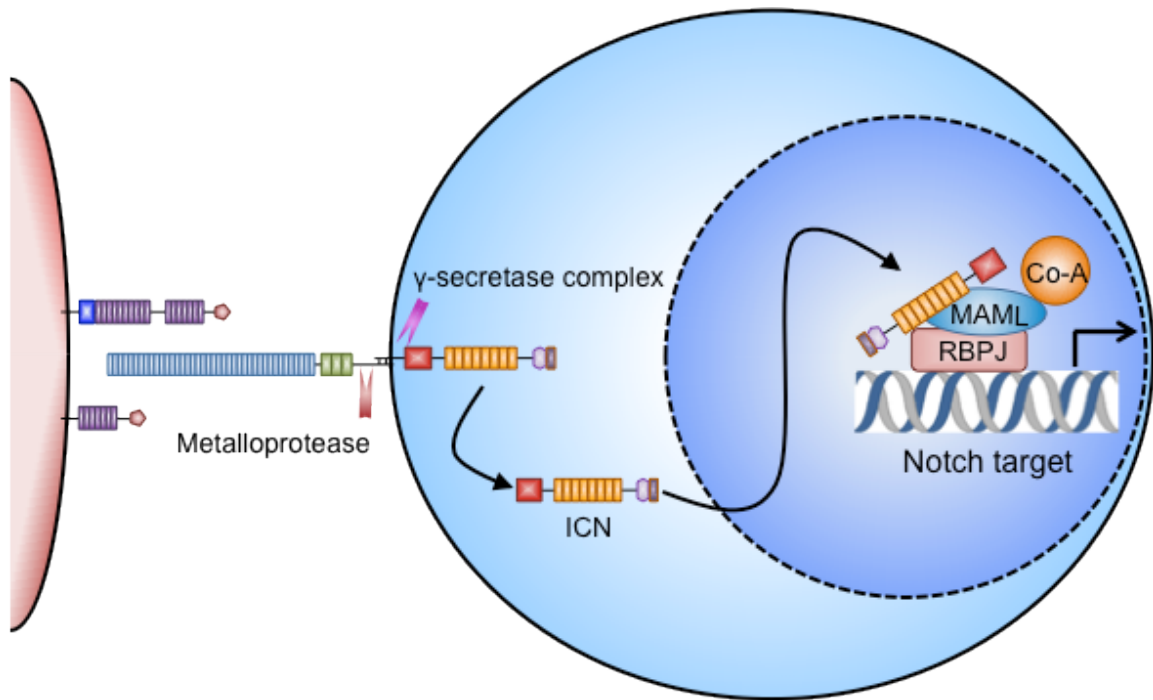


Figure 1-3: Schematic of Notch signaling. Notch signaling is initiated when a Notch receptor engages one of the four activating Notch ligands. This interaction exerts a mechanical force on the receptor, exposing a series of proteolytic cleavage sites. Metalloproteases and the γ -secretase complex then process the Notch receptor, liberating the intracellular Notch domain (ICN). Once released from the membrane, ICN is free to translocate to the nucleus where it interacts with its binding partners: RBPJ and MAML. The assembly of the Notch transactivation complex recruits co-activators (Co-A) that promote the transcription of Notch target genes.

Notch signaling and thymocyte development

Although Notch1, 2, and 3 are all expressed in developing thymocytes, only Notch1 is both necessary and sufficient for T cell lineage commitment. Overexpression of the ICN1 fragment of Notch1 in bone marrow progenitors causes ectopic T cell development and impairs B cell development, indicating a role for Notch in instructing the T rather than B fate decision (Figure 1-4) (Pui et al., 1999). Consistent with gain-of-function data, reciprocal loss-of-function experiments revealed that the inducible deletion of Notch1 or RBPJ or expressing a GFP-tagged, dominant-negative form of MAML (DNMAML) resulted in a severe block in T cell development at the DN stage and the accumulation of thymic B cells (Han et al., 2002; Maillard et al., 2004; Radtke et al., 1999). Conditional deletion models further mapped the stage-specific requirements for Notch signaling. Deletion of a floxed RBPJ gene at the DN2 stage, using Lck-cre, resulted in a developmental block at the DN3 stage, whereas use of a CD4-cre, which allows for deletion between the DN3 and DN4 stages, did not alter T cell development (Tanigaki et al., 2004). Moreover, the development of the DNMAML transgenic mouse provided the ability to track Notch inhibition at the single cell level *via* the GFP tag on DNMAML. Studies using these mice revealed that the developmental block observed in other loss-of-function models occurred in DN3a cells at the β -selection checkpoint. Importantly, when DN3 cells from Lck-cre x DNMAML mice are intrathymically injected into recipients, DNMAML expressing thymocytes failed to give rise to appreciable numbers of DP or SP cells, clearly illustrating an absolute requirement for Notch signaling in the DN compartment (Maillard et al., 2006). Together, these data favor a model in which Notch signaling is essential up until a functional TCR β is rearranged, but that persistent Notch signals are not essential thereafter. The findings discussed in this thesis will go on to

show that this transition from Notch-dependent to Notch-independent development at β -selection is also accompanied by a switch in the oncogenic potential of the Notch pathway as well as a shift in its activity as an lineage instructing factor to a signal integrating factor that potentiates multiple fates.

Notch signaling and the pre-TCR

The timing of the transition from Notch-dependent to Notch-independent development suggests cross-talk between pre-TCR and Notch signaling, and research has revealed significant interaction between these two signaling pathways. Chromatin immunoprecipitation (ChIP) and reporter assays have shown that Notch signaling governs pre-TCR signaling by regulating *Ptcra* gene expression, which encodes a surrogate TCR α (pTa) that complexes with a functionally rearranged β -chain to facilitate pre-TCR signaling (Bellavia et al., 2007; Reizis and Leder, 2002). In addition to directly activating *Ptcra* expression, the Notch pathway also indirectly promotes *Ptcra* gene expression. RBPJ shares a DNA binding motif with the transcription repressor Ikaros and data suggests there is the potential for these two proteins to compete for binding sites (Hsu et al., 1994; Ono et al., 1998). Signaling through Notch3 promotes the expression of the RNA binding protein HuD, which results in enhanced generation of Ikaros isoforms that lack the capacity to bind DNA, through alternative splicing. In turn, these isoforms disrupt the activity of full-length Ikaros and mediate enhanced RBPJ binding at pTa and increased Notch-dependent expression (Bellavia et al., 2007). Finally, Notch signaling is also implicated in promoting rearrangement of the *Tcrb* loci, suggesting that Notch might regulate pre-TCR signaling at the level of both pre-TCR α and TCR β expression (Ciofani et al., 2006; Wolfer et al., 2002).

While Notch signaling is a key regulator of the pre-TCR machinery, the resulting pre-TCR signals are essential for extinguishing *Notch1* expression and mediating the progression to Notch-independent development. From the DN1 stage up through the DN3a stage of thymocyte development, *Notch1* mRNA expression increases, and is regulated both by Notch1 itself and the E-proteins E2A and HEB (Ikawa et al., 2006; Yashiro-Ohtani et al., 2009). After β -selection, *Notch1* expression sharply decreases in DN3b cells and remains low for the remainder of thymic development (Taghon et al., 2006; Yashiro-Ohtani et al., 2009). Accordingly, *Notch1* expression is rapidly and robustly downregulated upon PMA and ionmycin stimulation of Rag2-deficient DN3 cells, demonstrating that pathways downstream of the pre-TCR are capable of directly regulating *Notch1* (Yashiro-Ohtani et al., 2009).

This interplay between pre-TCR signaling and *Notch1* expression is mediated by cross-talk of these two pathways with E-proteins. While Notch signaling is the primary driver of the T lineage program, E-proteins are essential for coordinating the kinetics of Notch activity during thymocyte development. *E2a* deficiency results in decreased expression of *Notch1* and *Notch3* in fetal thymocytes and causes a developmental block at the DN3 stage that can be rescued by overexpression of ICN1, placing Notch downstream of E2A (Ikawa et al., 2006). Moreover, E2A activity mirrors that of Notch1. E2A remains active until β -selection and pre-TCR signals promote the expression of the E-protein antagonist *Id3*, which disrupts E2A DNA-binding in later stages of development (Bain et al., 2001; Engel et al., 2001; Xi et al., 2006). Altogether, this creates a signal transduction circuit of a feedforward loop involving E2A-driven *Notch1* expression, followed by Notch1 autoregulation, which is then abolished by a negative feedback loop originating with pre-

TCR signaling and terminating with Id3 antagonism of E2A and downregulation of *Notch1* expression (Yashiro-Ohtani et al., 2009).

In addition to the direct cross-talk between Notch and pre-TCR signaling, there is significant interplay between the two pathways at the level of survival and proliferation signals in DN3 cells. Both pathways display significant overlap with the AKT pathway and AKT signals can bypass the requirement for both Notch and pre-TCR signaling at the DN3 stage. Use of myristoylated AKT (myr-AKT), a membrane tethered and constitutively active form of AKT, can partially bypass the requirement for Notch signaling during β -selection (Ciofani and Zuniga-Pflucker, 2005). Furthermore, deletion of the PI3K/AKT signaling antagonist, phosphatase and tensin homologue (PTEN), permits the development of thymocytes from DN3 to DP cells, even in the absence of IL-7 or pre-TCR signals (Hagenbeek et al., 2004). While AKT is downstream of pre-TCR signaling, Notch signaling does not directly activate the AKT pathway. Notch has been suggested to regulate AKT through multiple overlapping mechanisms. The canonical Notch target Hes1 binds the *Pten* locus and negatively regulates *Pten* in both normal and oncogenic T cell development (Palomero et al., 2007; Wong et al., 2012a). In a separate arm of Notch-AKT cross-talk, components of the mammalian target of rapamycin (mTOR) complex have been implicated as Notch pathway targets in the context of T-ALL (Chan et al., 2007; Lee et al., 2012). Finally, Notch has also been found to regulate the expression of receptors on thymocytes that engage the AKT pathway, such as the insulin-like growth factor 1 receptor and the IL-7Ra (Gonzalez-Garcia et al., 2009; Medyouf et al., 2011). Collectively these data support a model in which Notch and pre-TCR signaling both provide critical inputs into the AKT pathway at the DN3 stage, leading to a transition from a Notch-mediated to a TCR-driven survival program.

Although there is overlap between Notch and pre-TCR signaling, the two pathways are not redundant in thymocyte development. Multiple groups have found that pre-TCR and TCR signaling are incapable of compensating for a loss of Notch. In an *in vitro* T cell differentiation system, retroviral expression of TCR β or TCR $\alpha\beta$ could not support T cell development in the absence of Notch signals. Moreover, the same study found that anti-CD3e treatment or expression TCR β in conjunction with constitutively active Lck, Fyn, Ras, or PKC α all failed to bypass the requirement for Notch, demonstrating that the inability to overcome a lack of Notch signaling doesn't stem from a quantitative deficiency in pre-TCR signaling (Ciofani et al., 2004). Corroborating these *in vitro* findings, the DN3 block observed in Lck-cre x DNMA1 mice cannot be rescued by the enforced expression of *Tcrb* or *Tcra/b* transgenes (Maillard et al., 2006). Conversely, Notch signaling by itself is incapable of overcoming the loss of pre-TCR signaling. RAG2 deficient mice cannot generate the double-stranded breaks necessary for functional TCR β rearrangement and thus display a block at the β -selection checkpoint; overexpression of ICN1 in these mice fails to restore development into the DP stage. Similarly, overexpression of ICN1 in SLP76 deficient mice, an essential downstream component of pre-TCR signaling, fails to drive the development of DP cells (Allman et al., 2001).

While it remains unclear how these two pathways provide qualitatively distinct inputs into T cell differentiation, recent work has begun to clarify this issue. Detailed analysis of thymocyte expansion in the presence of cell cycle inhibitors has evinced an essential role for proliferation in the DN to DP progression. In the absence of Notch signaling, inducing proliferation by overexpression of either cell cycle machinery components or the Notch target *Myc* was capable of partially restoring *in vitro* development up to the DP

stage. In contrast, inducing proliferation by overexpressing cell cycle machinery was not sufficient to rescue development in the absence of a pre-TCR, as seen in Rag2 deficient thymocytes (Kreslavsky et al., 2012). Thus, the proliferative burst observed post- β -selection is an essential downstream function of Notch during T cell development, whereas the pre-TCR pathway provides distinct, cell-cycle-independent signals. Altogether, these findings indicate that Notch and pre-TCR signaling each provide essential and non-redundant inputs into thymocyte development.

Notch signaling and T-ALL

The robust pro-survival and proliferative signals provided by the Notch pathway during normal thymopoiesis also make it a potent oncogene. Indeed, Notch was first discovered through the analysis of T-ALLs with a (7;9) translocation that resulted in the fusion of *NOTCH1* on chromosome 9 to *TCRB* enhancer/promoter elements and the aberrant expression of a constitutively active, truncated nuclear Notch1 (Ellisen et al., 1991). When ICN1 is expressed in murine bone marrow progenitors, a rapid onset T-ALL emerges, demonstrating that Notch activation is sufficient for transformation (Figure 1-4) (Pear et al., 1996).

While originally identified in tumors containing a *TCRB* translocation, this mutation was later found to be rare. Instead, the predominant *NOTCH1* gain of function mutations, which are found in over 60% of human T-ALLs, occur *via* point and truncation mutations to the *NOTCH1* gene itself (Weng et al., 2004). The majority of *NOTCH1* mutations arise from point mutations to the NRR, which either destabilize the extracellular domain and permits ligand-independent cleavage of Notch1 and release of the ICN signaling peptide,

or they destroy the PEST domain, which prolongs the half-life of nuclear NOTCH1 (Weng et al., 2004). Both classes of mutations allow cells to both increase the level of Notch signal received and sustain Notch signaling in environments that no longer support it. Although this increased “portability” of Notch signaling may play a part in allowing transformed cells to escape the thymus, the dose of Notch signaling is a critical determinant of whether Notch acts as an oncogene or an effector of T cell development. Weak activating mutations to the NRR or PEST domain are sufficient to drive ectopic T cell development in transduced bone marrow cells and suppress B and myeloid differentiation, but fail to give rise to leukemia in the absence of additional genetic lesions. However, when multiple activating mutations to Notch are present or when ICN1 is expressed, the resulting signals promote both ectopic T cell development and leukemia (Chiang et al., 2008). Thus, normal T cell development must walk a tenuous line between the ability of Notch1 to initiate and drive the T cell program and the capacity of Notch to act as a potent oncogene.

The oncogenic potential of Notch signaling likely stems from its positioning as a key regulator of multiple pro-survival and pro-proliferative pathways. ChIP-on-chip profiling after Notch inhibition in gamma-secretase inhibitor (GSI) treated T-ALL cell lines revealed that more than 40% of Notch-responsive loci were effectors of cell metabolism and protein biosynthesis (Palomero et al., 2006). Among these genes, c-Myc has been identified as a critical Notch target in T-ALL (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006). Expression of c-Myc rescues T-ALL cell lines from Notch withdrawal and c-Myc inhibitors block the ability of ICN1 to rescue T-ALL cell lines from GSI treatment, demonstrating that c-Myc is both necessary and sufficient for Notch1's leukemogenic functions (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006).

In addition to c-Myc, Notch signaling has also been suggested to regulate the NF- κ B and AKT pathways. Ectopic expression of ICN1 in bone marrow progenitors promotes the nuclear localization of NF- κ B, the expression of NF- κ B components, and the expression of NF- κ B target genes. Although inhibition of NF- κ B impairs the growth of Notch1-dependent human T-ALL cell lines, NF- κ B is not sufficient to drive T-ALL (Vilimas et al., 2007). Given that NF- κ B signaling is necessary but not sufficient for T-ALL, NF- κ B may act in collaboration with Notch1 to promote c-Myc expression, as studies have identified NF- κ B binding sites in the *Myc* promoter (Park and Wei, 2003). In parallel to its effects on NF- κ B, Notch signaling has also been implicated in regulating the AKT pathway in cancer. The Notch-Hes1-PTEN axis seen during thymocyte development is an important mediator of Notch driven T-ALL. Loss of PTEN is a frequent occurrence in GSI-resistant T-ALLs and is sufficient to rescue the survival and proliferation defects observed in GSI treated, Notch-dependent T-ALL cell lines, indicating AKT is an important downstream effector of Notch in T-ALL (Palomero et al., 2007). Accordingly, AKT signaling has been found to be a critical downstream mediator of Notch1-driven glycolysis (Landor et al., 2011). Moreover, multiple reports have linked Notch signaling to the AKT pathway member mTOR. Loss of Rictor, a component of mTORC2, significantly increases median survival and reduces leukemic cell organ infiltration in a murine model of Notch1-induced T-ALL (Lee et al., 2012). Corroborating this finding, GSI treatment or expression of DNMA1L in T-ALL cell lines results in the inhibition of multiple components of mTOR signaling that could be rescued by expression of ICN1 (Chan et al., 2007). Altogether these data indicate that while c-Myc is the primary mediator of Notch-driven T-ALL, Notch signaling regulates multiple pathways with known oncogenic roles and likely promotes transformation by the overlapping pro-survival and pro-proliferative inputs from these signals. Chapter 3 will review data illustrating that the oncogenic activity of Notch

is lost after β -selection, opening the way for future studies to identify the molecular and cellular contexts in which Notch can transform cells.

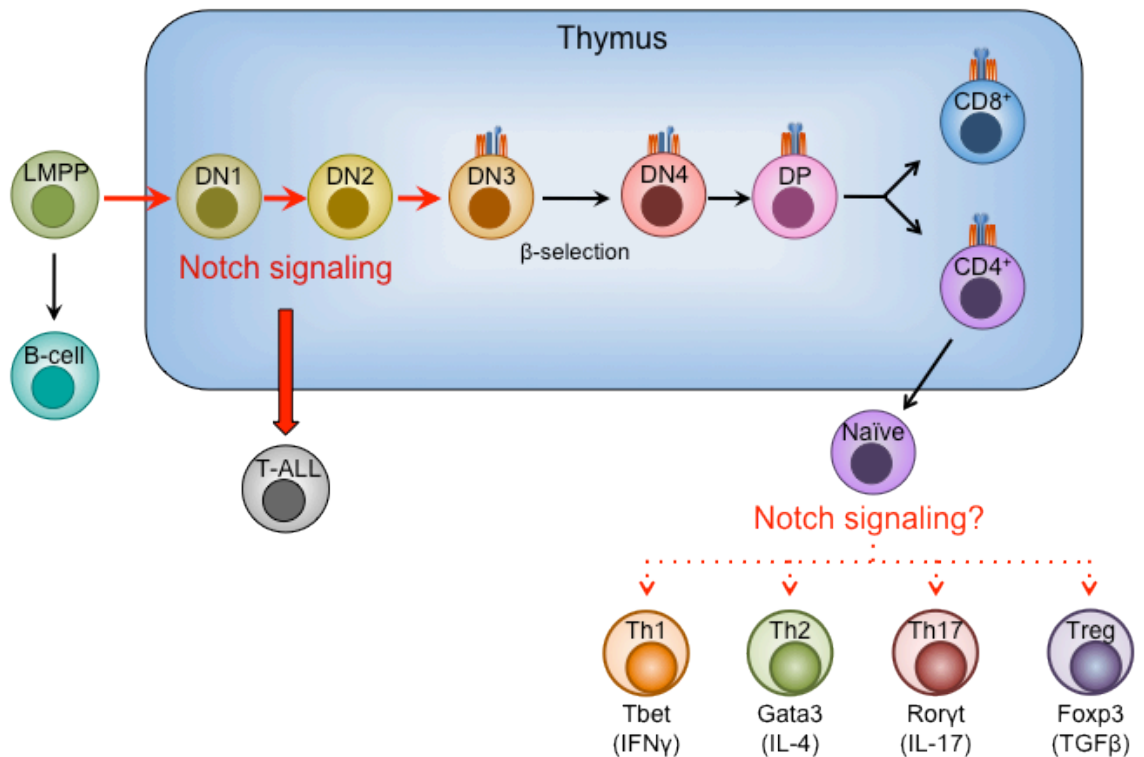


Figure 1-4: Notch signaling and T lineage development. Notch signaling instructs T cell development in LMPP's at the expense of B cell differentiation. The pathway remains active from the DN1 through the DN3 stage of thymocyte development, where it is a critical regulator of proliferation and cell survival. Hyper-activation of Notch signaling during this period results in the potent induction of T-ALL. Notch signaling is extinguished once a thymocyte successfully passes through the β -selection checkpoint and the remainder of thymic development is understood to be Notch-independent. When a mature, naïve CD4⁺ T cell enters the periphery and becomes activated, Notch signaling is reinitiated and understood to play an important role in subsequent Th cell differentiation decisions; however it remains unclear how or whether Notch instructs the fate of these various subsets.

Peripheral CD4⁺ T cell differentiation

The Th1-Th2 paradigm and the linear instruction model

Though CD4⁺ T cell diversity has long been appreciated, our ability to delineate the discrete boundaries that define individual Th cell populations has waxed and waned as our understanding of the complex relationship between these subsets has evolved. Initial efforts to classify Th cell subsets ranged from the use of cell surface antigens (Arthur and Mason, 1986; Bottomly, 1988; Morimoto et al., 1985a; Morimoto et al., 1985b), to designations based on short- and long-term survival post-thymectomy (Kappler et al., 1974), and even classifying Th cells based on the use of antigen recognition versus B cell idiotype recognition (Bottomly and Mosier, 1979; Janeway et al., 1977). While these methods were effective in fractionating the CD4⁺ T cell compartment, they did not yield consistent functional immune correlates (Mosmann and Coffman, 1989).

Following the establishment of the first functional T cell hybridoma lines and the relentless efforts to identify and characterize the growing world of “lymphokines,” Tim Mossman and Richard Coffman were equipped with the tools necessary to establish the modern Th cell paradigm (Kontinen et al., 1978; Mosmann and Coffman, 1989). The observation that T cell hybridoma lines separated into two distinct groups based on cytokine production and that this pattern of cytokine production could predict the mode of B cell help given revolutionized our understanding of host immunity (Mosmann et al., 1986). Moreover, the findings that these disparate Th cell cytokine phenotypes were stable over periods as long as 18 months and failed to interconvert provided compelling evidence that these populations were distinct lineages (Mosmann and Coffman, 1989). Subsequent studies demonstrating that this method of Th cell fractionation was sufficient

to explain whether certain inbred mouse strains were susceptible to specific classes of pathogens, based on the cytokine bias of Th cell from these mice, illustrated that cytokines and Th cell subtype – not just antigen/MHC specificity – were critical determinants of pathogen-specific immune responses (Else and Grencis, 1991; Heinzel et al., 1989; Locksley et al., 1987; Sadick et al., 1987; Scott et al., 1988).

Once the precursor-product relationship for both Th1 and Th2 cells was mapped back to a common progenitor – the thymectomy-sensitive, naïve T cell – efforts became focused on understanding how a bifurcation in fate choice could arise in a developmentally mature cell type (Seder and Paul, 1994). It was appreciated early that exogenous factors played a key role in differentiation. The signature cytokines of each Th cell subtype were found to act in an autocrine manner to reinforce their corresponding program and inhibit the adoption of the alternate phenotype (Fernandez-Botran et al., 1988; Gajewski and Fitch, 1988). Furthermore, the observation that IL-12 was necessary and sufficient to induce Th1 differentiation *via* STAT4 (Gazzinelli et al., 1993; Hsieh et al., 1993; Kaplan et al., 1996b; Thierfelder et al., 1996) and that IL-4 *via* STAT6 was necessary and sufficient for Th2 differentiation (Betz and Fox, 1990; Hou et al., 1994; Kaplan et al., 1996a; Le Gros et al., 1990; Swain et al., 1990) suggested that these two population differentiated in response to unique signaling modules, despite shared pathways downstream of the TCR and co-stimulation. Finally, the discovery of Tbet and Gata3 as factors that were not only obligate for Th1 and Th2 differentiation, respectively, but could impose Th1 or Th2 phenotypes on already differentiated cells cemented the notion that discrete, mutually-exclusive Th cell subsets existed and solidified a linear instruction model of Th cell differentiation that has come to dominate the field (Figure 1-5) (Szabo et al., 2000; Zheng and Flavell, 1997).

Many aspects of this model have been repeatedly supported by findings in the literature, demonstrating its predictive utility, particularly *in vitro* and within the bi-polar Th1-Th2 paradigm. The linear instruction hypothesis contends that differentiated cells will have distinct functional programs, governed by unique transcriptional networks downstream of unique exogenous factors that are self-reinforcing and antagonistic to alternate fates. Indeed, cytokine production by Th cells is often dominated by either IFN γ or IL-4 in a large number of classically used infection models (Else and Grencis, 1991; Heinzel et al., 1989; Locksley et al., 1987; Sadick et al., 1987; Scott et al., 1988). Moreover, the ability of Th cells to provide host protection in these models requires animals to have the capacity to express the appropriate fate-specifying cytokines or cytokine receptors, the signal transducers that are downstream of those cytokine receptors, and a discrete and specific network of transcription factors regulated by those signal transducers (Altare et al., 1998; Mattner et al., 1996; Rosas et al., 2006; Stamm et al., 1999; Urban et al., 1998). Not only does each Th cell subset require these transcription factor networks, but enforced expression of one network has been shown to destabilize the activity of others (Szabo et al., 2000; Zheng and Flavell, 1997). Altogether, the strength of the linear instruction hypothesis has led to its use as a predictive mechanistic model for experimental findings for nearly a quarter century.

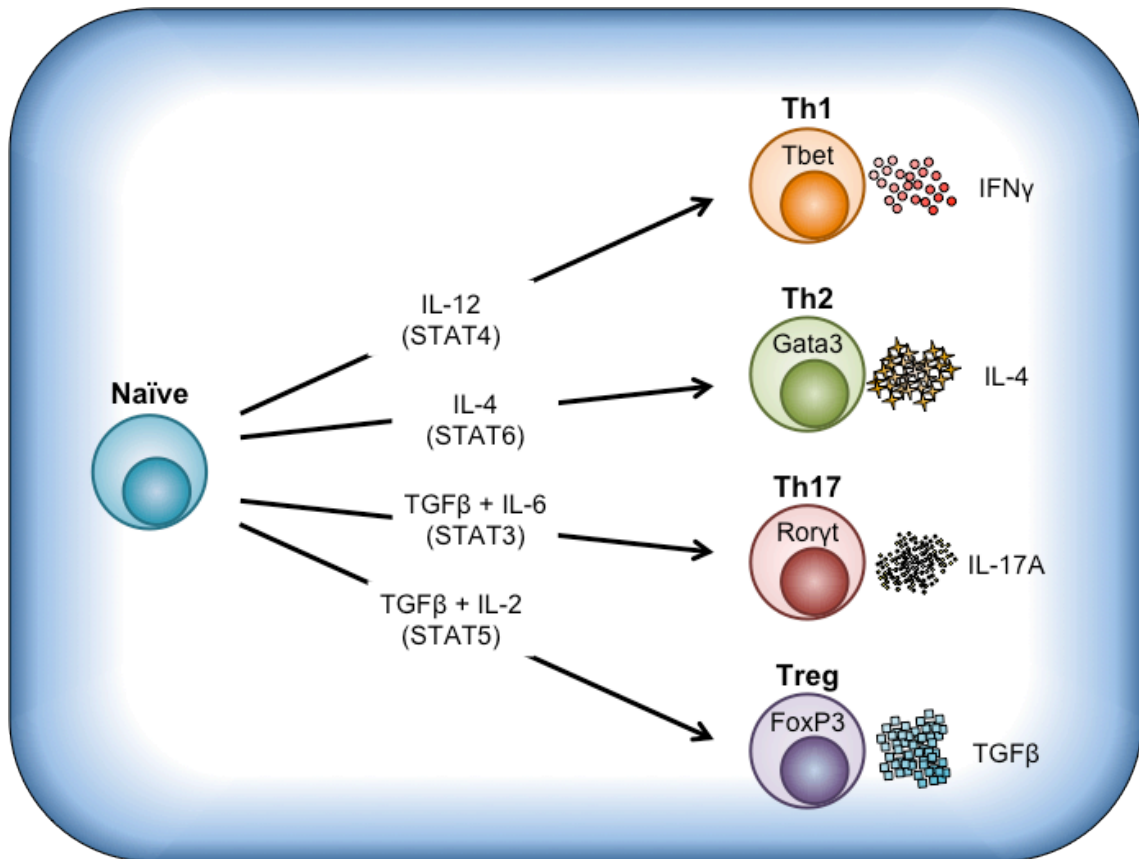


Figure 1-5: The linear instruction model of Th cell differentiation. Naïve CD4⁺ T cells differentiate in response to unique cytokine mixtures soon after activation. Cytokines stimulate the activation of signal transducers that in turn promote the expression of lineage defining “master regulator” transcription factors. The master regulators imprint lineage identity, as defined by the production of a signature cytokine, and restrict potential for alternate Th cell fates. STAT4 acts downstream of IL-12 to induce Tbet expression and impose the Th1 identity. IL-4 signaling activates STAT6, resulting in Gata3 expression and Th2 differentiation. A combination of the immunoregulatory factor TGF β and the immunoinflammatory cytokine IL-6 results in STAT3-dependent Ror γ t expression. Finally, the Treg cell identity is imprinted by the expression of Foxp3, which is induced by TGF β plus IL-2 and requires STAT5.

More lineages, more problems: the linear instruction model challenged

Despite the durability of this hypothesis, many experimental observations are largely inconsistent with its implicit predictions. Researchers have long been aware that many human Th cell populations fail to fall within these mutually exclusive bins, with the exception of instances of severe inflammation (Bucy et al., 1994; Kelso, 1995; Maggi et al., 1988; Romagnani, 1991). Even in the *in vitro* systems employed by Mosmann and Coffman, cells failed to show cytokine production or transcription patterns on a single cell level that were consistent with single, dominant transcriptional programs (Bucy et al., 1994). More recently, the identification of IL-17A producing Th17 cells (defined by Ror γ t and induced in the presence of TGF β plus IL-6) and TGF β producing Treg cells (defined by Foxp3 and induced in the presence of TGF β plus IL-2) has presented the model's greatest challenge (Bettelli et al., 2006; Brunkow et al., 2001; Chatila et al., 2000; Fontenot et al., 2003; Ivanov et al., 2006). Although researchers have shown that these populations can be generated *in vitro* based on first principles of the linear instruction hypothesis, the identification of Th17 and Treg cells has called several key aspects of this model into question.

An essential component of the linear instruction hypothesis is that each Th cell identity is defined by the expression of unique cytokine profiles and the identity of each subset is actively maintained by the expression of a single "master regulator." Initial findings from Th17 and Treg studies were consistent with these axioms, however the existence of Th cell populations that produce multiple lineage defining cytokines and transcription factors is now widely appreciated. Th17 cells have been shown not only to have the capacity to produce both IL-17A and IFN γ concurrently, but also the ability to lose IL-17A expression

and solely produce IFN γ (Algood et al., 2007; Bending et al., 2009; Chen et al., 2007; Lee et al., 2009; Shi et al., 2008). Consistent with cytokine production data, Th17 cells have also been found to simultaneously express Tbet and Ror γ t (Annunziato et al., 2007; Shi et al., 2008). In addition to the mixed Th cell profile observed in Th17 cells, Treg cells have also been found to exhibit features of Th cell duality. Given their shared requirement for TGF β in differentiation, it is not surprising that Th cells can co-express Foxp3 and Ror γ t and that Foxp3⁺ cells can produce IL-17A (Xu et al., 2007; Yang et al., 2008). Perhaps more unanticipated, Tregs are now known to have the potential to express the Th1 and Th2 “master regulators,” Tbet and Gata3. Foxp3⁺ cells have been found to co-express Tbet and IFN γ and these factors are understood play an important role in Treg localization and repressor function during type-1 inflammatory responses (Koch et al., 2009; Oldenhove et al., 2009; Sawitzki et al., 2005; Stock et al., 2004). Similarly, not only can Tregs co-express Foxp3 and Gata3, but Gata3 expression in Tregs has also been found to play an essential role in Treg biology and may even act to limit inflammatory Th cell differentiation (Rudra et al., 2012; Wang et al., 2011; Wohlfert et al., 2011). Further complicating the role of Gata3 as the “master regulator” of Th2 differentiation, Gata3 expression is also required for the differentiation of IL-9 producing Th9 cells (Dardalhon et al., 2008; Veldhoen et al., 2008). While IL-9 secreting cells can produce IL-4 like Th2 cells, Gata3⁺IL-4⁻ Th9 cells can exist as a population independent of Th2 cells and there are even IL-17A⁺ cells that have the capacity to produce IL-9 (Elyaman et al., 2009; Licona-Limon et al., 2013). Altogether, these data are fundamentally incompatible with the linear instruction hypothesis and are more indicative of a continuum of states and/or overlapping functional programs.

These findings not only suggest that Th cells are capable of simultaneously supporting multiple programs to generate nuanced responses, but also illustrate a second irreconcilable axiom of the linear instruction hypothesis: that the unique cytokine signatures of Th cells are representative of discrete lineages. By definition, a cell lineage represents a distinct developmental progression resulting in a stable functional grouping of cells. The extent to which functional groups can interconvert dictates whether multiple cell populations represent true lineages (i.e. T versus B lineage cells) or whether they instead represent modular functional programs within a single lineage (i.e. IgE versus IgG producing B cells). As described above, Th cells have the capacity to change cytokine production and transcriptional network profiles. The fact that this had only been observed with regard to the Th17 and Treg phenotypes had previously been cited as evidence to argue that Th1 and Th2 cells did in fact represent distinct cell lineages (Zhou et al., 2009b). However, recent work demonstrates that interferons and IL-12 can reprogram Th2 cells into a hybrid Gata3⁺Tbet⁺ state during viral infection, evincing that these two Th cell types are in fact not stable, mutually exclusive lineages, but rather are reprogrammable, semi-stable functional modules (Hegazy et al., 2010). Importantly, this has not only been shown functionally but also at the level of epigenetic regulation. ChIP-seq analysis of Th cell populations has revealed that, rather than shutting down transcriptional programs for alternate fates as has been observed in similar analyses of related lineages, differentiated Th cells maintain signature genes for alternate programs in a poised chromatin conformation (Cedar and Bergman, 2011; Wei et al., 2009). Thus Th cell type flexibility appears to be a normal feature of CD4⁺ T cell activation.

In light of the mounting evidence that Th cell differentiation is unlikely to operate under the linear instruction model, it is worthwhile reconsidering the Th subtype-specific

instructional roles that have been ascribed to a wide variety of exogenous cues that are received by activated CD4⁺ T cells. Activated CD4⁺ T cells integrate a multitude of external signals in addition to cytokines, including signals derived from the TCR and co-stimulatory molecules. Although originally described as instructive, these molecules have all been found to play a role in the differentiation of multiple Th cell subsets.

Antigen dose and altered peptide ligand studies have been used to argue that TCR signal strength instructs Th1 or Th2 differentiation: with high levels of TCR stimulation suggested to instruct Th1 differentiation and weak TCR stimulation suggested to instruct Th2 differentiation (Constant and Bottomly, 1997; Zhu et al., 2010). In stark contrast to these peptide-based studies, *in vivo* infection models have shown that high levels of infection correlate with higher levels of Th2 cytokine production, whereas lower levels of infection result in increased Th1 cytokine production, with pathogens ranging from *Leishmania major* to *Trichuris muris* giving the same result (Bancroft et al., 1994; Bretscher et al., 1992; Nicholson et al., 1995; Sarzotti et al., 1996). While there are obviously many confounding factors that affect our ability to interpret these *in vivo* infection studies, it is clear that antigen dose itself is neither necessary nor sufficient to instruct Th cell type choice, in contrast to cytokines in these models. Moreover, O'Garra and colleagues observed that both very high and low doses of ovalbumin peptide promote Th2 differentiation, suggesting that the influence of TCR signaling on Th cell differentiation is not simply a matter of signal strength (Hosken et al., 1995). Finally, the Th2-biasing activity of altered peptide ligands that diminish TCR signal strength is highly dependent on the cytokine environment, with higher doses IL-2 being sufficient to override these effects (Tao et al., 1997). Hence, while TCR signaling can influence both

Th1 and Th2 differentiation under certain conditions, it does not display activities consistent with an instructional pathway.

Like TCR signaling, the linear instruction hypothesis has been applied to the role of co-stimulatory molecules in Th cell differentiation. The B7 family of co-stimulatory molecules – consisting of CD28, CTLA4, and ICOS – play an essential role in T cell activation and have also been argued to instruct Th cell differentiation (Constant and Bottomly, 1997; Zhu et al., 2010). Although multiple studies initially implicated CD28 and CTLA4 as instructive signals, these molecules were found to play unbiased roles in Th cell differentiation and instead are critical tuners of T cell activation (Constant and Bottomly, 1997; Zhu et al., 2010). ICOS has similarly been suggested to instruct Th2 differentiation, with ICOS deficient mice reported to display severely impaired IL-4 production (Dong et al., 2001). However, subsequent studies have illustrated a complex role for ICOS in Th cell biology. Inhibition of ICOS signaling has been found to both positively and negatively regulate IL-4 and IFN γ production in *in vivo* models as well as promote or protect against the Th17-mediated disease model experimental autoimmune encephalomyelitis, depending on the timing of ICOS inhibition (Harada et al., 2003; Rottman et al., 2001; Sporici et al., 2001). Moreover, ICOS signaling has also been shown to be critical in Treg differentiation and/or function, where it has even been found to be necessary for limiting Th2 responses (Akbari et al., 2002; Herman et al., 2004). Collectively, these findings indicate that, while both TCR and co-stimulation are critical inputs that can influence Th cell differentiation, they do not behave instructionally and their diverse activities in multiple Th cell populations highlights the existence of a large, shared signaling network amongst the Th cell subsets.

As discussed above, outside of the role that highly defined mixtures of cytokines play in Th cell differentiation *in vitro*, the vast majority of evidence suggests that Th cell differentiation operates as a series of overlapping functional modules coordinated by a large, shared network of signaling pathways that are integrated to provide semi-stable effector states. Within this framework, the need for molecules that help integrate these inputs into coherent outputs becomes just as great as the need for cytokines in enforcing specific functional outcomes. Although initial efforts were spent attempting to fit the Notch signaling pathway into the linear instruction model, this thesis contends that the Notch pathway instead acts as an unbiased integrator of environmental differentiation cues.

Notch signaling in peripheral CD4⁺ T cell differentiation

Notch as an instructor of Th1 versus Th2 cell differentiation

Given the Notch pathway's unequivocal instructional role in T lineage commitment from normal hematopoietic progenitors and in launching the T-ALL program from dysregulated precursors, it is not surprising that this classical instructional paradigm was also applied to its role in Th cell differentiation. With only Th1 and Th2 cells appreciated at the time, Amsen and colleagues hypothesized that Notch signaling instructed this process much like it did in other bipotential fate decisions. They went on to find that while Notch pathway did display some characteristics of an instructional pathway, the outcome of Notch signaling depended on the type of presenting Notch ligand. Delta-like ligands expressed on MHC-bearing fibroblasts were shown to instruct Th1 differentiation and limit Th2 differentiation, while Jagged ligands were found to instruct Th2 differentiation. Intriguingly, they also observed that LPS treatment could induce Notch

ligand expression on dendritic cells (DCs) in a TLR4 dependent manner, and that Jagged and Delta-like ligands could each be selectively expressed by stimulation with unique stimuli, fitting with a prevailing model that the caliber of Th cell responses is patterned by the activating stimuli antigen presenting cells receive. Despite these findings, RBPJ deficiency only affected the ability of LPS treated DC's to promote IL-4 production, even though this stimulus induced the expression of both Delta-like and Jagged ligands. Reciprocally, expression of ICN1 in T cells only resulted in increased Th2 differentiation (Amsen et al., 2004). Further complicating these findings, another study using a similar Notch ligand expression system found no instructive capacity for either Jagged or Delta-like ligands. Instead, they argued for a model in which Notch behaves selectively to promote Th1 or Th2 differentiation, but in a manner entirely dictated by upstream cytokine signaling (Ong et al., 2008). There is now also evidence that Delta-like ligands are key regulators of Th2 responses *in vivo* and that Jagged ligands can promote Th1 differentiation (Jang et al., 2010; Le Friec et al., 2012; Mukherjee et al., 2014). Nevertheless, this seminal study established the ligand instruction model, which would become the prevailing hypothesis for the complex role Notch signaling plays during Th cell differentiation.

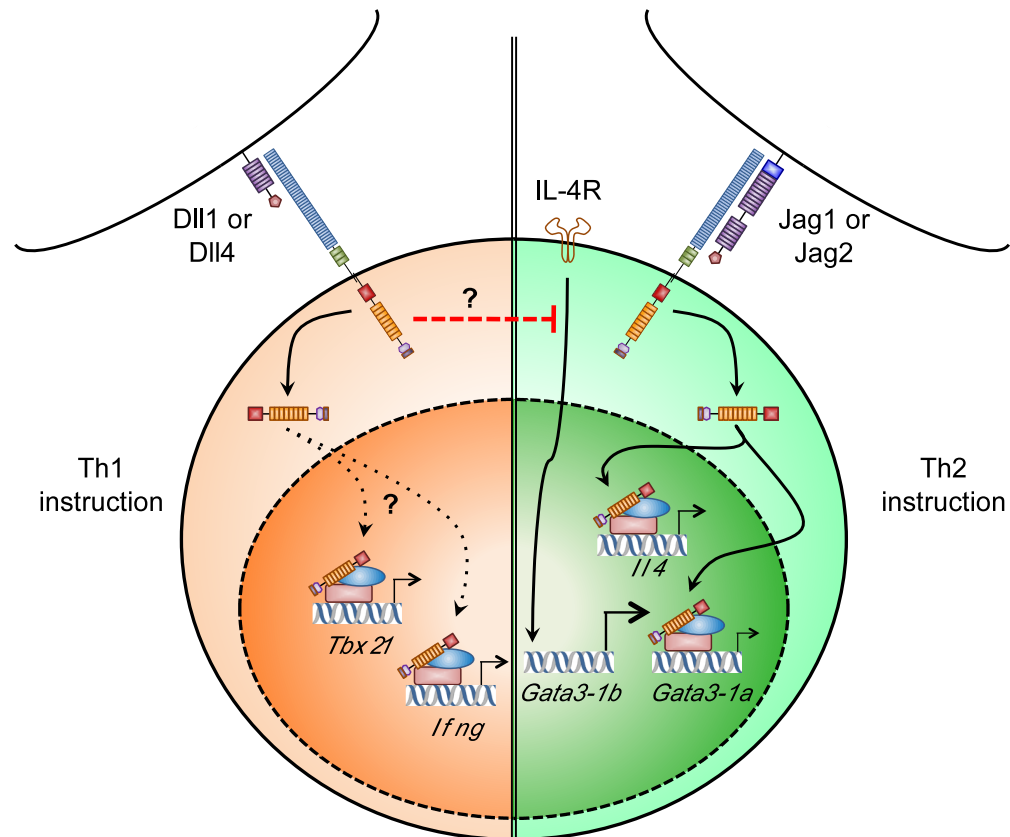


Figure 1-6: The Notch ligand instruction model. Notch ligands have been proposed to differentially instruct either Th1 or Th2 differentiation. In this model, Delta-like ligands are thought to instruct Th1 differentiation by promoting the transcription of *Tbx21* and *Ifng* and by inhibiting Th2 differentiation downstream of the IL-4R, though the transcriptional and inhibitory activity of the Notch pathway in this setting has not formally been shown. Similarly, Jagged ligands are thought to instruct Th2 differentiation by directly promoting the transcription of *Il4* and the transcription of *Gata3* from exon 1-a. However, the *Gata3-1a* transcript represents a minor fraction of the total *Gata3* mRNA, with the *Gata3-1b* isoform being the dominant transcript, regulated downstream of IL-4R signaling.

Subsequent studies similarly argued for an instructional model, however they disagreed with regard to which Th cell subset Notch signaling promoted. Oral administration of γ -secretase inhibitors (GSI) or pre-treatment of T cells with GSI was reported to severely inhibit IFN γ production by Th1 differentiated cells, while having no effect on IL-4 production under both Th2 and non-polarizing conditions. The same study also found that GSI treatment inhibited *Tbx21* (Tbet) transcription, but not *Gata3*, and expression of ICN1 could enhance IFN γ production and *Tbx21* transcription, even in Th2 differentiated cells, *via* binding to the *Tbx21* promoter (Minter et al., 2005). In addition to reported roles for Notch1 in promoting Th1 differentiation, Notch3 has also been suggested to instruct the development of this Th subset. Exogenous expression of ICN3 in T cells resulted in enhanced Th1 differentiation at the expense of Th2 differentiation, and the same effect was observed upon treating mice or T cells with an activating Dll1-Fc fusion protein (Maekawa et al., 2003). Further highlighting a role for Notch signaling in Th1 differentiation, LPS treated CD8⁻ DC's have been shown to promote Th1 differentiation independent of IL-12 production. This phenomena has been suggested to be due to increased expression of Dll4 on CD8⁻ DC's that in turn results in Dll4-dependent Th1 differentiation, however the requirement for Notch signaling in the receiving T cells was never tested (Skokos and Nussenzweig, 2007).

In spite of evidence supporting Notch as a selective instructor of Th1 differentiation, multiple *in vitro* and *in vivo* loss-of-function studies have contended the contrary. In addition to data from RBPJ deficient T cells discussed above, work using CD4-cre x DNMA1L^{FL/FL} T cells also found that Th2 differentiation was severely impaired when Notch signaling was inhibited, while Th1 differentiation remained intact. Moreover, these mice were resistant to *L. major* infection, a Th1-dependent model, but susceptible to *T.*

muris infection, which requires a Th2-dependent immune response for protection (Tu et al., 2005). Corroborating these findings, CD4-cre x RBPJ^{FL/FL} mice exhibit a significantly impaired Th2 response and an enhanced Th1 response following immunization and restimulation with *Schistosoma mansoni* egg extract and have also been found to be resistant to *L. major* infection (Amsen et al., 2007; Auderset et al., 2012). Not only has loss of Notch signaling been found to impair Th2 differentiation, but expression of ICN1, and to a lesser extent ICN2-4, robustly and selectively promotes Th2 differentiation *in vitro* (Amsen et al., 2007; Fang et al., 2007).

At the transcriptional level, Notch is thought to mediate this instructive capacity by directly regulating the *Il4* and *Gata3* loci. Notch1 binds to and directly regulates *Il4*, as seen in ChIP analysis and luciferase assays (Amsen et al., 2004; Fang et al., 2007). This activity is dependent on the 3' *Il4* enhancer, HS5: when Notch signaling is enforced in transgenic T cells bearing an *Il4* minilocus, transcription from the minilocus is only enhanced when constructs contain the HS5 (Amsen et al., 2007). Notch regulation of *Il4* also requires Gata3 activity, but can occur in the absence of STAT6. Expression of ICN1 in Gata3-deficient T cells or in the presence of a dominant-negative Gata3 failed to promote IL-4 protein expression (Amsen et al., 2007; Fang et al., 2007). Moreover, while expression of ICN1 alone had a minimal capacity to enhance IL-4 production in Th1 cultures, co-expression of Gata3 and ICN1 synergistically promoted IL-4 production in Th1 cells, in a STAT6-independent fashion (Fang et al., 2007). While these findings established Notch as a regulator of the Th2-defining cytokine, they also raise questions over the Th2 instructing capacity of the Notch pathway. Although ICN1 can induce IL-4 expression in activated T cells and even reprogram Th1 cells, Notch signaling itself was not sufficient for either of these functions and required the potent stabilizer of Th2

identity, *Gata3*. In fact, Notch potentiates Th1 differentiation in *Gata3*^{-/-} T cells (Amsen et al., 2007). These data may instead suggest that Notch lacks any instructional activity alone and rather cooperates with other regulators of Th cell programming.

Not only has Notch been shown to regulate *Il4* in cooperation with *Gata3*, but it has also been found to directly regulate *Gata3* itself. *Gata3* can be expressed as two transcripts through two different variants of exon 1: *Gata3-1a* and *Gata3-1b* (Asnagli et al., 2002). As shown by both ChIP and EMSA, the Notch transcriptional activation complex can associate with both exons 1a and 1b. In addition, GSI treatment inhibits the accumulation of Notch at both regions, indicating that Notch has the potential to dynamically modulate *Gata3* expression through both transcripts (Amsen et al., 2007; Fang et al., 2007). Although expression of DNMA1L decreased transcription at both sites after seven days of culture, expression of ICN1 was sufficient to potently drive *Gata3-1a* transcription after only 36 hours and failed to activate transcription at *Gata3-1b*, suggesting that Notch only regulates expression of transcripts initiated at exon 1a (Fang et al., 2007). Despite compelling data indicating that the Notch pathway can directly regulate *Gata3* expression, its inability to drive expression at *Gata3-1b* does not fit with an instructional role for Notch. While both transcripts are expressed in Th2 cells, *Gata3-1b* is expressed within the first 24 hours of activation, whereas expression from *Gata3-1a* doesn't begin until day 3 post activation (Asnagli et al., 2002; Yu et al., 2009). Moreover, *Gata3-1b* transcripts are much more abundant than the *Gata3-1a* variant (Yu et al., 2009). Thus, although Notch can regulate *Gata3-1a*, the kinetics and magnitude of expression are incompatible with an instructive role for Notch in Th2 differentiation, *via* *Gata3*. Chapter 2 will discuss data suggesting that Notch does not instruct Th cell

differentiation, but instead acts to simultaneously promote the differentiation of Th1, Th2, and Th17 cells by sensitizing cells to exogenous differentiating cues.

Notch and non-classical Th cell subsets

While studies conflicted over whether Notch instructed Th1 versus Th2 differentiation, the ligand instruction model was made even more tenuous by additional reports implicating Notch in other Th cell populations. Suggesting a role for Notch in Th17 biology, recombinant-Dll4 stimulation can promote Th17 differentiation *in vitro* and TLR stimulated APCs can enhance Th17 differentiation in a Dll4 and Notch dependent manner. This effect was suggested to be mediated by Enhanced STAT3 signaling as well as by the ability of the Notch transactivation complex to bind to the *Rorc* (Roryt) promoter (Mukherjee et al., 2009). A second corroborating study similarly showed that siRNA knockdown of Notch1 or GSI treatment of human and mouse T cells inhibited *in vitro* Th17 differentiation. This study not only confirmed Notch binding to *Rorc* by ChIP, but also identified *Il17a* as a probable direct target (Keerthivasan et al., 2011). Findings from both these studies indicate that Notch signaling regulates Th cell differentiation beyond the Th1/Th2 paradigm and that Dll4 can promote the differentiation of non-Th1 subsets, which is not consistent with predictions from the ligand instruction model.

In addition to Th17 differentiation, Notch signaling has now been suggested to regulate the putative Th cell subsets Th9 and T follicular helper (Tfh) cells (defined by Bcl6 expression and IL-21 production) (Crotty, 2011). CD4-cre x Notch1^{FL/FL}Notch2^{FL/FL} mice display impaired IL-9 secretion under both Th9 and Th17 conditions. Conversely, Jagged ligand stimulation or ICN1 expression results in enhanced IL-9 production. This

effect on IL-9 is direct: Notch binds to the *IL9* promoter, where it synergizes with Smad3 to induce transcription, as seen in ChIP and luciferase assays (Elyaman et al., 2012). While the data supporting a role for Notch in Th9 biology is compelling, it is unclear the extent to which Notch is involved in the differentiation or the function of this subset. Moreover, the extent to which Th9 cells represents a *bona fide* Th cell subset or IL-9 production is a functional module that can be overlaid on top of Th2 and Th17 differentiation remains undetermined (Murphy and Stockinger, 2010).

Like Th9 cells, it is unclear if Tfh cells should be characterized as a distinct Th cell subset or as a program parcel that can further specialize Th1 and Th2 cells (Murphy and Stockinger, 2010); nonetheless recent work suggests that Notch signaling may be involved in Tfh biology. CD4-cre x Notch1^{FL/FL}Notch2^{FL/FL} display severely impaired Tfh responses after immunization with peptide, *L. mexicana*, or *S. mansoni* eggs. Mixed bone marrow chimeras similarly displayed reduced Tfh differentiation in immunization models, however the presence of WT cells partially rescued this defect, suggesting this is not entirely a cell autonomous phenotype. The finding that Notch signaling modulates the balance of *Bcl6* and *Blimp1* (a transcription factor known to antagonize Bcl6 activity) in activated T cells offers one potential explanation for this observation, but this was shown to be an indirect effect. Further complicating this study, CD4-cre x Notch1^{FL/FL}Notch2^{FL/FL} mice have compromised germinal center architecture and cell number following immunization or infection and are known to have defects in IL-4 production (Auderset et al., 2013). Given Tfh cells require normal germinal center function to properly differentiate, it is unclear whether these Tfh defects are caused by or are responsible for complications in germinal center formation (Crotty, 2011). Until the relationship of Tfh cells to the other known Th cell subsets is established, it will be

difficult to resolve whether these findings truly indicate a role for Notch in Tfh differentiation or if they instead result from impaired Th1 or Th2 differentiation due to loss of Notch signaling.

While the role Notch signaling plays in inflammatory Th cell differentiation is disputed, there is no greater clarity as to how Notch influences Treg biology. Both Notch1 and Notch 3 have been suggested to promote Treg differentiation. Lck-cre x ICN3^{FL/FL} mice display a nearly twenty-fold expansion of CD25⁺Foxp3⁻ cells, but also exhibit a roughly three-fold expansion of CD25⁺Foxp3⁺ cells. Moreover, these cells demonstrate elevated suppressor function. This expanded Treg population is lost when ICN3-transgenic mice are crossed to *Ptcra*^{-/-} mice, indicating that these cells are likely naturally occurring Tregs. It is worthwhile noting that these mice also develop T-ALL, making it difficult to determine whether these effects are secondary to additional complications in these mice (Anastasi et al., 2003; Campese et al., 2009). A second study found that GSI pretreatment of T cells or expression of an antisense Notch1 transgene resulted in decreased Treg differentiation *in vitro*. However, cleaved Notch1 protein was still present in significant quantities upon GSI treatment and Notch1 antisense expression, suggesting that the phenotypes observed cannot solely be explained by an absence of Notch signaling. The same study also found that Notch1 can bind to the Foxp3 promoter and GSI treatment depleted Notch1 from this region, however histone acetylation at both the Foxp3 promoter and an enhancer was unaffected and the consequence of this binding on transcription was never evaluated (Samon et al., 2008). In contrast to these two reports, multiple *in vivo* studies have found that inhibition of Notch signaling results in increased Treg differentiation. In models of graft-versus-host disease (GvHD) and experimental autoimmune encephalomyelitis (EAE), inhibition of Notch signaling

ameliorates disease and results in decreased inflammatory T cell differentiation coupled with enhanced Treg differentiation (Bassil et al., 2011; Sandy et al., 2013; Tran et al., 2013; Zhang et al., 2011). Not only do these disease models highlight a role for Notch in antagonizing Treg differentiation, but the observation that there are no steady-state developmental or inflammatory defects in mice with a conditional loss of Notch signaling in peripheral T cells suggests that Notch cannot be an essential positive regulator of Treg differentiation (Amsen et al., 2007; Amsen et al., 2004; Tu et al., 2005). Chapter 3 will discuss new findings indicating that Notch signaling acts to destabilize Treg differentiation, with data from CD4-cre x ICN1^{FL/FL} mice showing that activation of Notch in T cells leads to lethal autoinflammation, consistent with a negative regulatory role for Notch in Treg differentiation.

CHAPTER 2: Notch simultaneously orchestrates multiple helper T cell programs independently of cytokine signals

Abstract

Two models are proposed to explain Notch function during helper T (Th) cell differentiation. One argues that Notch instructs one Th cell fate over the other, whereas the other posits that Notch function is dictated by cytokines. Here we provide a detailed mechanistic study investigating the role of Notch in orchestrating Th cell differentiation. Notch neither instructed Th cell differentiation nor did cytokines direct Notch activity, but instead, Notch simultaneously regulated the Th1, Th2, and Th17 cell genetic programs independently of cytokine signals. In addition to regulating these programs in both polarized and non-polarized Th cells, we identified *Ifng* as a direct Notch target. Notch bound the *Ifng* CNS-22 enhancer, where it synergized with Tbet at the promoter. Thus, Notch acts as an unbiased amplifier of Th cell differentiation. Our data provide a paradigm for Notch in hematopoiesis, with Notch simultaneously orchestrating multiple lineage programs, rather than restricting alternate outcomes.

Introduction

The T helper cell program adopted by a naïve CD4⁺ T cell is instructed both by extracellular molecules, such as cytokines, and intracellular molecules, such as the Th1, Th2, and Th17 cell transcription factors, Tbet, Gata3, and Ror γ t respectively. Notch has also been proposed to mediate Th cell differentiation, where it functions to relay intercellular signals from the membrane to the nucleus in order to instruct Th cell differentiation (Amsen et al., 2009).

As reviewed in Chapter 1, compelling cases have been made for Notch involvement in both Th1 and Th2 cell differentiation. Manipulating Notch ligand mediated stimulation of CD4⁺ T cells preferentially instructed Th1 or Th2 cell programs, suggesting that individual Notch ligands have different instructive capacities (Amsen et al., 2004; Maekawa et al., 2003; Okamoto et al., 2009). Loss of function studies also demonstrated that Notch instructed the Th1 cell program *in vitro* and promoted the CD4⁺ T cell IFN γ response in a murine GVHD model (Minter et al., 2005; Skokos and Nussenzweig, 2007; Zhang et al., 2011). In contrast, other reports showed that Notch was required to instruct the Th2 but not the Th1 cell program (Amsen et al., 2007; Amsen et al., 2004; Fang et al., 2007; Kubo, 2007; Tu et al., 2005). More recently, Notch was found to regulate the Th17 cell signature genes *Il17a* and *Rorc*, suggesting the bi-potential instructional model may not be sufficient to explain Notch function in Th cell differentiation (Keerthivasan et al., 2011; Mukherjee et al., 2009). While the instructional model posits that ligands direct Notch function during Th cell differentiation, an alternative model argues that Notch target gene selectivity is dictated by upstream cytokine signals (Ong et al., 2008). Despite the differences between these models, both contend that Notch has the capacity

to discriminately activate different Th cell programs. Thus, the paradox remains: how can such a basic signaling module selectively drive the differentiation of multiple distinct lineages?

In order to address these controversies, we investigated the molecular mechanisms by which Notch orchestrates Th cell differentiation. We find that Notch neither initiates a single helper T cell program nor do cytokine signals dictate the outcome of Notch signaling. Instead, Notch simultaneously regulates the Th1, Th2, and Th17 genetic programs independently of cytokine signals. Even under strong polarizing conditions, Notch directly regulates critical effectors of Th1 Th2, and Th17 cell differentiation. In addition to *Il4*, *Tbx21*, *Gata3-1a*, *Il17a*, and *Rorc*, we identify *Ifng* as a direct Notch target. Notch regulates *Ifng* by binding to a highly conserved RBPJ motif in the *Ifng* CNS-22 and synergizes with Tbet activity at the *Ifng* promoter. These data support a model in which Notch integrates and amplifies cytokine-derived signals, instead of acting as a transcriptional driver or a downstream accessory of cytokines. Not only do our data unify the disparate data on Notch and Th cell differentiation but they also offer an alternative view of Notch function in the hematopoietic system, whereby Notch reinforces multiple fates rather than restricting alternate outcomes.

Results

Notch signaling is dispensable for Th2 cell initiation during Trichuris muris infection.

We previously showed that CD4⁺ T cells expressing the pan-Notch inhibitor dominant negative mastermind (DNMAML), which binds the Notch:RBPJ dimer but fails to transactivate, do not mount an effective Th2 cell response against the intestinal helminth *Trichuris muris* and fail to clear infection with normal kinetics (Tu et al., 2005). The outcome of *T. muris* infection depends on the balance of Th1 cells, which are responsible for chronic infection, and Th2 cells, which are required for parasite expulsion and resistance to infection (Artis et al., 2004; Blackwell and Else, 2001; Cliffe and Grencis, 2004; Cliffe et al., 2005; Else et al., 1994). While Notch was necessary for optimal Th2 cell-dependent immunity in this infection model, it remained unclear whether Notch was essential to initiate Th2 cell differentiation or instead, was required to generate the optimal balance of Th1 and Th2 cells. To test this, *CD4-cre* (CC) and *CD4-cre* x DNMAML^{FL/FL} (CCD) mice were infected with *T. muris* and CCD mice were treated with neutralizing anti-IFN γ mAbs for the duration of infection. If Notch were required to initiate Th2 cell differentiation, anti-IFN γ treated CCD mice should remain susceptible to *T. muris* infection. Alternatively, if Notch played a greater role in generating an optimal Th2 cell response, then IFN γ blockade should be sufficient to relieve any inhibitory effects of a suboptimal Th1:Th2 ratio on infection-induced Th2 differentiation.

As expected, mesenteric lymph node cells from the control CC mice displayed robust IL-4, IL-5 and IL-13 responses upon restimulation (Figure 2-1A). Consistent with our previous findings, CCD mice demonstrated impaired Th2 cell cytokine responses (Figure 2-1A). In contrast, CCD mice receiving anti-IFN γ mAb treatment restored IL-4, IL-5, and

IL-13 production and diminished IFN γ production, suggesting that Notch was not required to initiate Th2 responses (Figure 2-1A). In agreement with the cytokine data, CCD mice displayed an impaired protective *T. muris* specific IgG1 response and an elevated non-protective *T. muris* specific IgG2c response. In contrast, anti-IFN γ mAb treated CCD mice recovered parasite specific IgG1 and showed a trend towards decreased IgG2c (Figure 2-1B). Similarly, while CCD mice displayed decreased serum IgE, anti-IFN γ mAb treatment restored this response (Figure 2-1C). Furthermore, histologic analysis of intestinal sections revealed that anti-IFN γ mAb treatment rescued the goblet cell mucin response in CCD mice (Figure 2-1D). Finally, anti-IFN γ mAb treatment restored the ability of CCD mice to expel parasites with kinetics comparable to infected CC control mice by 21 days post infection (Figure 2-1E). Collectively, these data demonstrate that Notch is not essential to initiate Th2 cell responses *in vivo*, and instead suggest that Notch functions to optimize the response.

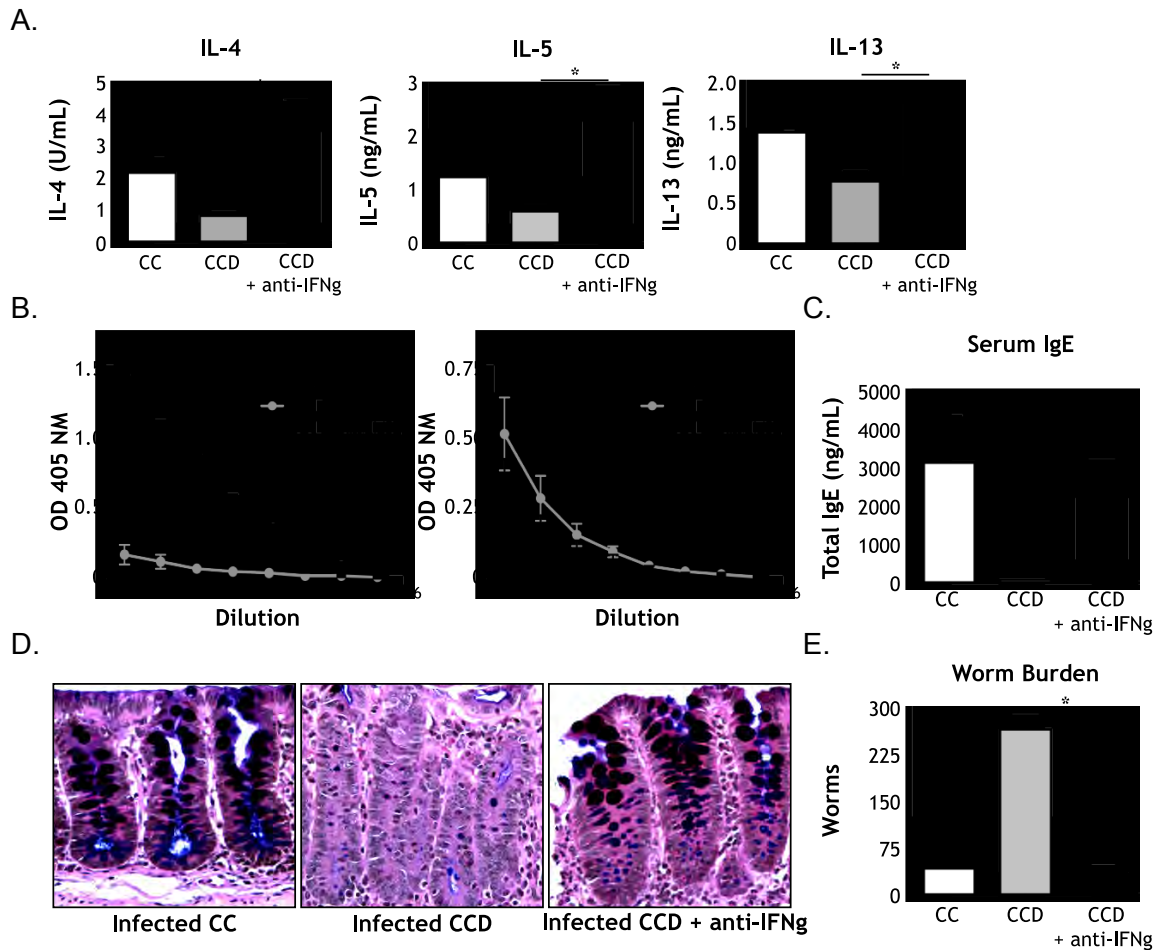


Figure 2-1: Notch signaling is dispensable for Th2 initiation during *Trichuris muris* infection. (A) At day 21, MLN from *T. muris* infected CC, CCD, and CCD + anti-IFN γ mice were stimulated with anti-CD3 for 72 hours and cytokine levels were measured by ELISA. (B) Serum *T. muris* specific IgG1 and IgG2c and (C) total IgE were measured by ELISA from infected mice, at day 21. (D) Goblet cells in gut sections were detected by mucin staining. (E) Worm burden at day 21 post-infection. *, $P < 0.05$

Persistent Notch signaling is required to maintain the Th1 and Th2 cell programs.

To further test whether Notch is required to maintain an optimal Th2 cell response, we investigated the effect of inhibiting Notch subsequent to Th2 cell differentiation. For these studies, we developed an in vitro differentiation system in which Notch could be inhibited at different times by addition of a gamma secretase inhibitor (GSI) following activation of CD4⁺ T cells. This system also provided the opportunity to test the requirement for Notch in Th1 cell differentiation. Importantly, Notch inhibition by GSI did not affect T cell activation, proliferation, or cell numbers, even after prolonged exposure (Figure 2-2A-C), in contrast to a recent report (Helbig et al., 2012). To look at Notch specific effects and exclude autocrine cytokine effects, cells were cultured in the presence of neutralizing IL-4 and IFN γ antibodies.

To test whether Notch was required post-initiation to maintain both the Th1 and Th2 cell programs, naïve CD4⁺ T cells were activated in the presence of irradiated splenocytes. After 5 days, cells were restimulated and treated with either DMSO or GSI and left in culture for an additional 2 days (day 7) before harvest. When looking at Th1 cell signature genes, both mRNA and protein for Tbet and IFN γ were significantly lower after 2 days of GSI treatment (Figure 2-3A-C). Similarly, GSI decreased mRNA and protein expression of the Th2 signature genes, *Gata3* and *Il4* (Figure 2-3A-C).

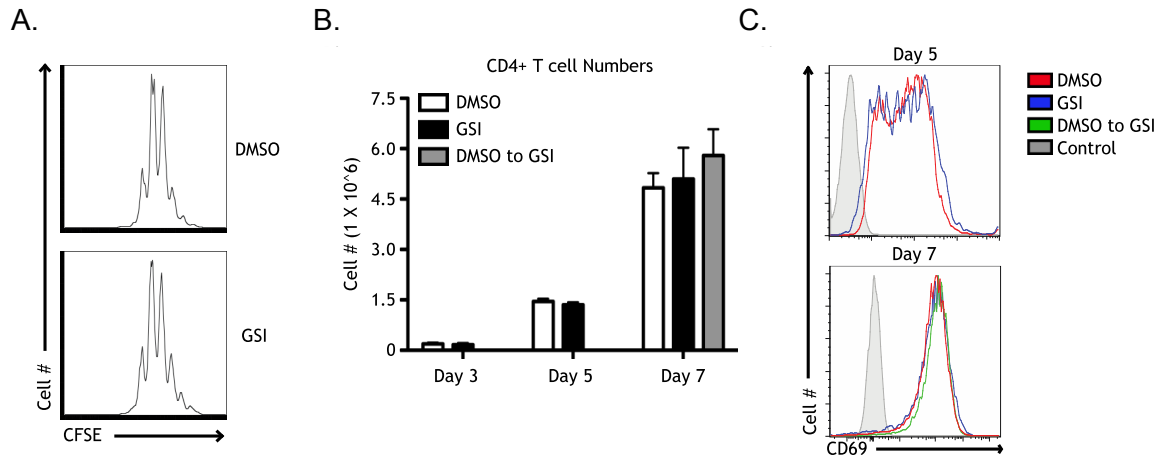


Figure 2-2: GSI treatment does not affect T cell activation. (WT naive CD4⁺ T cells were CFSE labeled and then stimulated with irradiated splenocytes (1:5), anti-CD3e (1 μ g/mL), and anti-CD-28 (1 μ g/mL), cultured in media containing 5 ng/mL IL-2, 20 μ g/mL anti-IL-4, and 20 μ g/mL anti-IFN γ and treated with either DMSO or 1 μ M GSI. On day 5, cells were washed, restimulated, and cells previously cultured in DMSO were either replated in DMSO or 1 μ M GSI, while cells previously cultured in GSI were maintained in GSI. (A) FACS analysis of cell proliferation on day 3 post-activation. (B) Cell numbers on days 3, 5, and 7 post-activation. (C) FACS analysis of CD69 expression on cells days 5 and 7 post-activation. Data are represented as mean \pm SEM. Related to Figure 2.

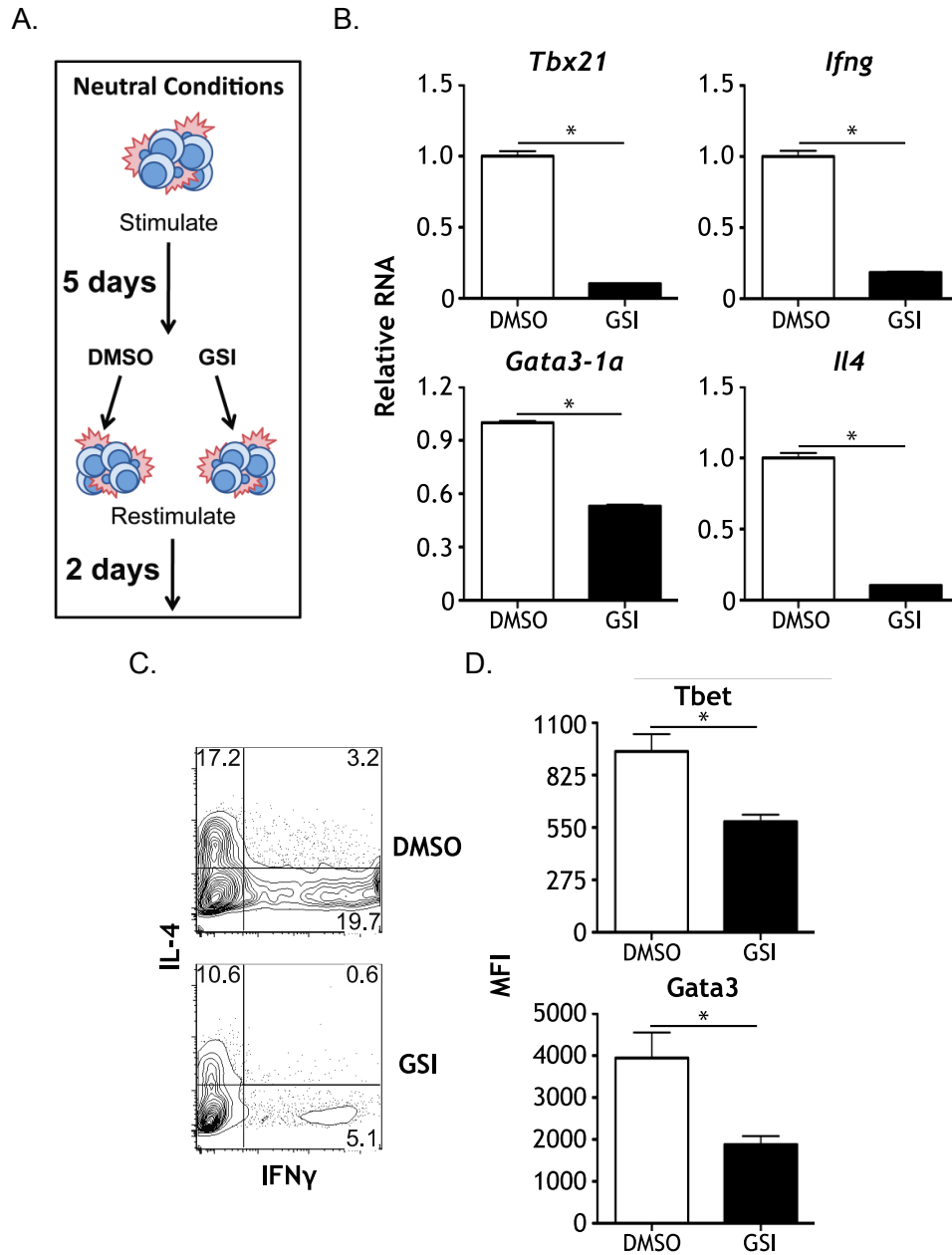


Figure 2-3: Persistent Notch signaling is required to maintain the Th1 and Th2 programs. (A) Schematic of experimental design. WT naive CD4⁺ T cells were stimulated with irradiated splenocytes, anti-CD3 ϵ , and anti-CD28, and cultured under neutral conditions. After 5 days, cells were restimulated as above and replated in media containing either DMSO or 1 μ M GSI. After 2 days (day 7 post-activation), (B) RNA was then harvested and analyzed by qPCR. Cells were analyzed for (C) cytokine production and (D) transcription factor expression by intracellular FACS. *, $P < 0.05$

While our data show that persistent Notch signaling is required to maintain the Th1 and Th2 cell programs post-initiation, it remained unclear whether the activity of Notch on Th1 and Th2 cell targets was restricted to early patterning events or if Notch was capable of re-activating target genes late after T cell stimulation. To address this, naïve CD4⁺ T cells were activated in the presence of either DMSO or GSI, under neutralizing conditions. After 5 days, cells were washed, restimulated, and returned to either DMSO or GSI to test whether cells previously treated with GSI could recover cytokine and transcription factor expression. While cells treated with GSI for all 7 days of culture showed impaired Tbet and IFN γ induction, cells treated with GSI only for the first round of stimulation recovered Tbet and IFN γ responses (Figure 2-4A-C). Similarly, IL-4 and GATA3 expression decreased when cells were treated with GSI for all 7 days and recovered when GSI was removed (Figure 2-4A-C). Our findings indicate that Notch is capable of promoting Th1 and Th2 signature gene expression both at early and late time points following stimulation and that inhibiting Notch at either the beginning of T cell stimulation or at later time points represses both Th1 and Th2 cell differentiation. Furthermore, we repeatedly observed that expression of both Th1 and Th2 cell program genes and proteins were suppressed in the same population upon Notch inhibition, suggesting that Notch concurrently regulates both programs. Collectively, these data show that Notch functions in Th cell differentiation do not require upstream signals from polarizing cytokines and demonstrate that the activity of Notch on its targets is not kinetically restricted.

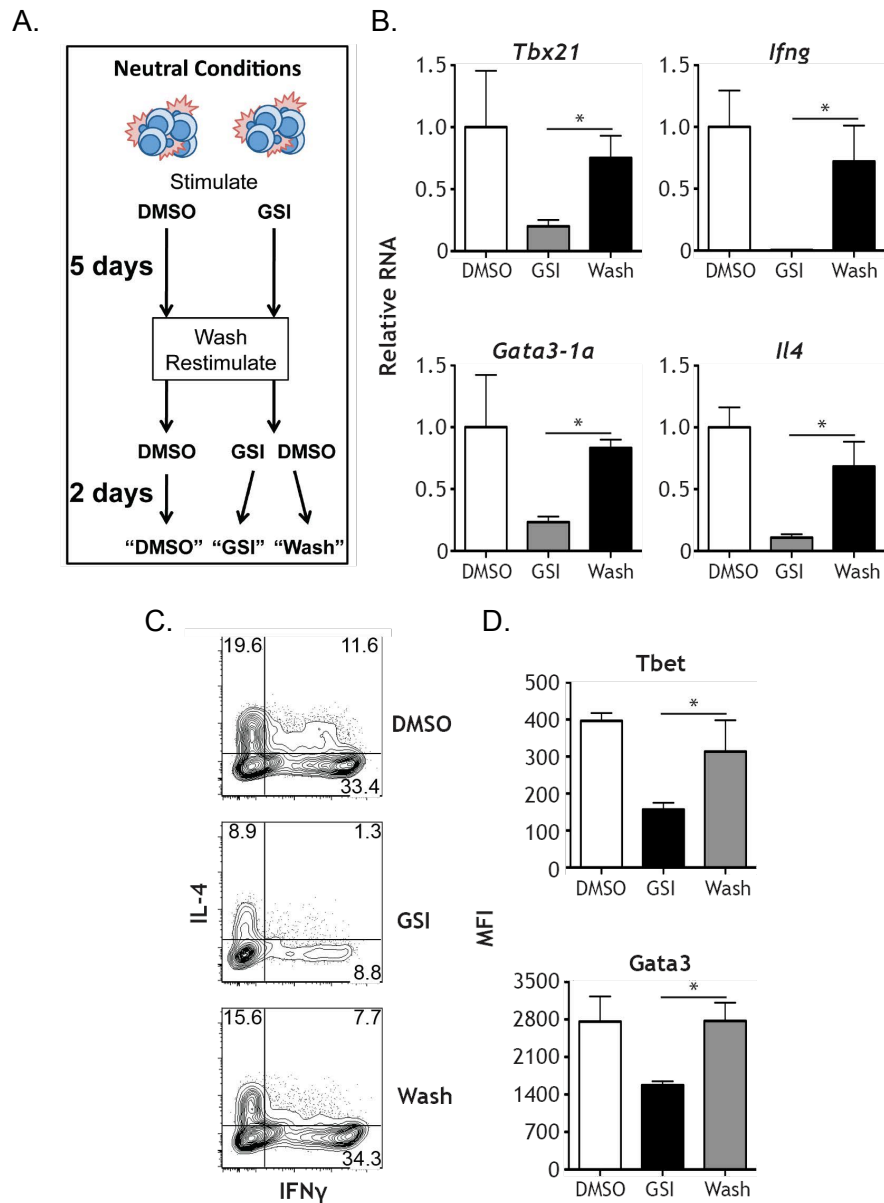


Figure 2-4: The activity of Notch signaling during T cell differentiation is not restricted to early activation events. (A) Schematic of experimental design. WT naive CD4⁺ T cells were stimulated and cultured as above for 5 days in media containing either DMSO or GSI. Cells were then washed and restimulated under the same conditions, with cells previously cultured in DMSO being replated in media containing DMSO and cells previously treated with GSI being replated in either DMSO or 1 μ M GSI. After 2 days of restimulation, (B) RNA was then harvested and analyzed by qPCR. Cells were analyzed for (C) cytokine production and (D) transcription factor expression by intracellular FACS. *, $P < 0.05$

Notch concurrently regulates both the Th1 and Th2 programs.

Although both Th1 and Th2 cell signature genes were sensitive to GSI, it was important to show that the effects were Notch-specific as GSI has Notch-independent effects. Furthermore, Notch-independent, Presenilin-dependent effects on cytokine production were reported for both Th1 and Th2 cell types (Ong et al., 2008). To test whether the changes we observed were Notch-specific, we utilized mice containing two floxed alleles of dominant negative mastermind (DNMAML^{FL/FL}), a potent and specific GFP-tagged pan-Notch inhibitor.

Tat-Cre treated YFP^{FL/FL} and DNMAML^{FL/FL} naïve CD4⁺ T cells were stimulated under neutralizing conditions. Use of the *Tat*-Cre peptide to acutely induce DNMAML expression minimized potential compensation for Notch signaling by other pathways. As expected, YFP⁺ T cells produced IL-4 and IFN γ (Figure 2-5A). Consistent with observations using GSI treatment, DNMAML expressing T cells displayed a marked reduction in the fraction of cells that produced IL-4 and IFN γ confirming that Notch regulates both IL-4 and IFN γ production in the same cell population (Figure 2-5A). In contrast to the GSI data, analysis of Tbet and GATA3 protein in DNMAML expressing cells revealed reduced expression of Tbet, but not GATA3 (Figure 2-5B). Notch had been shown to regulate *Tbx21* in a GSI-dependent manner and directly regulate *Il4* via a 3' enhancer; however, the GATA3 result was unexpected as Notch directly regulates *Gata3* transcription by binding to the *Gata3-1a* promoter in primary CD4⁺ T cells (Amsen et al., 2007; Amsen et al., 2004; Fang et al., 2007; Minter et al., 2005). When transcripts for both Th1 and Th2 signature genes were analyzed by qPCR, all four (*Il4*, *Gata3-1a*, *Ifng* and *Tbx21*) were expressed at lower levels in DNMAML expressing cells (Figure 2-5C). The observed differences in GATA3 protein expression between GSI and DNMAML

treatment were likely due to the finding that GSI suppressed transcripts from both *Gata3-1a* and *Gata3-1b*, whereas DNMAHL only suppressed the *Gata3-1a* transcript, which accounts for a minor fraction of total *Gata3* transcripts (Yu et al., 2009) (Figure 2-6A-B). These data indicate that Notch likely exerts its primary effect on the Th2 program through its regulation of *Il4*. It is important to note that while DNMAHL expression had no impact on cell proliferation (Figure 2-6C), we did observe a minor increase in apoptosis as measured by Annexin-V (Figure 2-6D). While this subtle increase cannot account for the marked reduction in cytokine transcript and protein observed in DNMAHL expressing cells, it suggests that a role for Notch signaling in cell survival may also contribute to these defects.

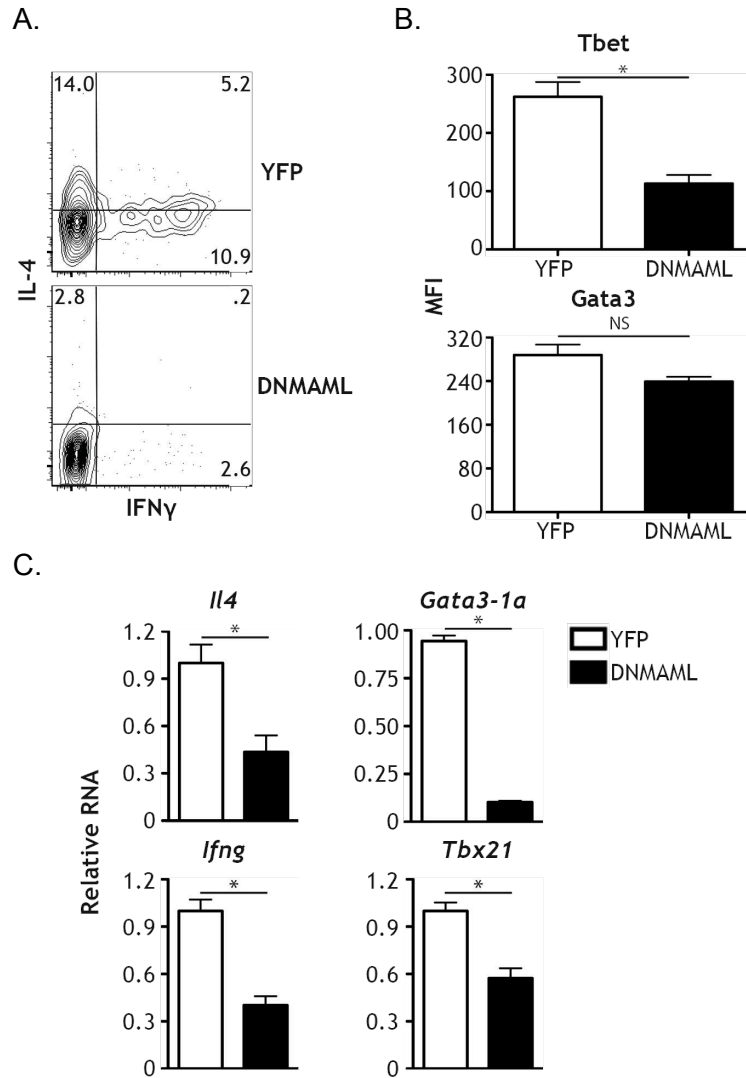


Figure 2-5: Notch signaling is required for the expression of both Th1 and Th2 signature genes. YFP^{FL/FL} or DNMAHL^{FL/FL} CD4⁺ T cells were Tat-cre treated and rested for 24 hours in media containing 100 ng/mL IL-7. Naive CD4⁺ T cells were then FACS sorted and stimulated with irradiated splenocytes, anti-CD3ε, and anti-CD28, and cultured under neutral conditions. After 5 days, (A) cytokine production was measured by intracellular FACS, (B) Tbet and Gata3 protein was measured by intracellular FACS, and (C) RNA was analyzed by qPCR. *, P < 0.05

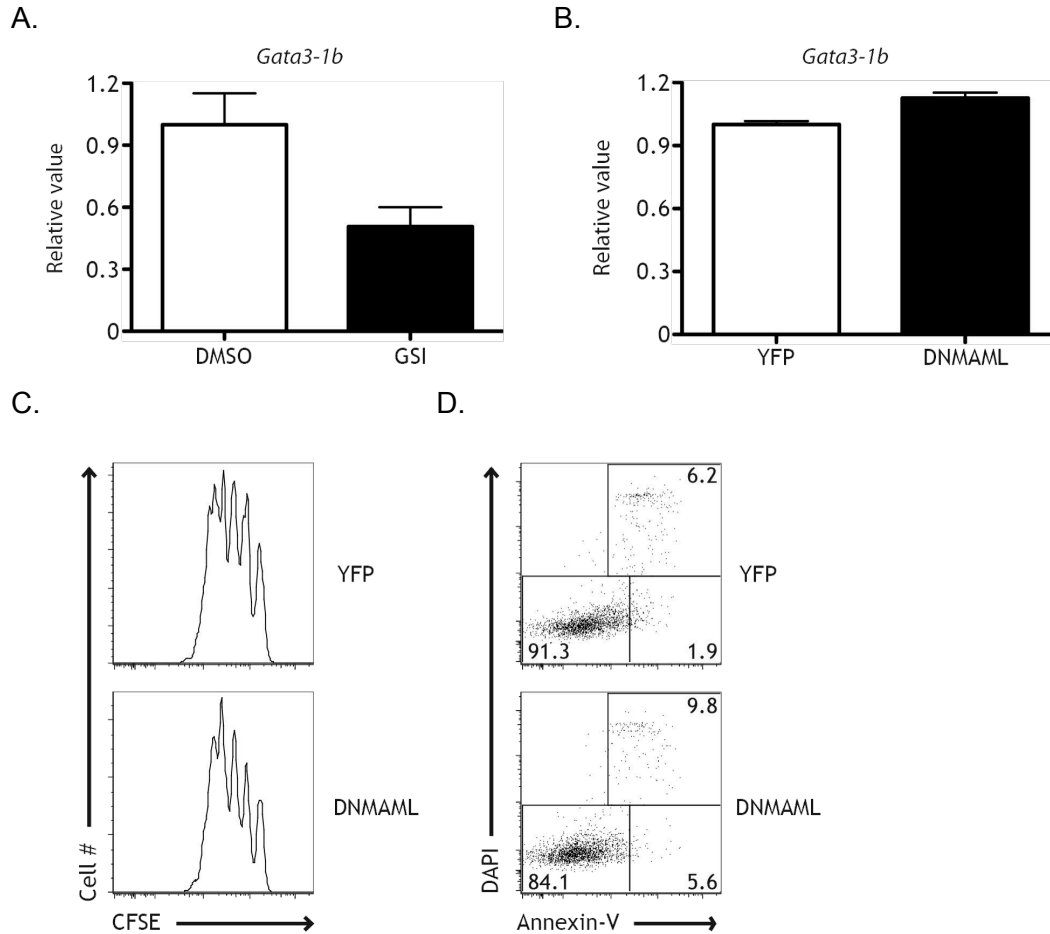


Figure 2-6: DNMA M expression does not affect *Gata3-1b* transcription or cell viability. (A) WT naïve CD4⁺ T cells were CFSE labeled and then stimulated with irradiated splenocytes (1:5), anti-CD3e (1 µg/mL), and anti-CD-28 (1 µg/mL), cultured in media containing 5 ng/mL IL-2, 20 µg/mL anti-IL-4, and 20 µg/mL anti-IFNγ and treated with either DMSO or 1 µM GSI. RNA was harvested on day 5 post-activation and analyzed by qPCR. (B) YFP^{fl/fl} or DNMA M^{fl/fl} CD4⁺ T cells were Tat-cre treated and rested for 24 hours in media containing 100 ng/mL IL-7. Naïve CD4⁺ T cells were then FACS sorted and stimulated with irradiated splenocytes (1:5), anti-CD3e (1 µg/mL), and anti-CD-28 (1 µg/mL), and cultured in media containing 5 ng/mL IL-2, 20 µg/mL anti-IL-4, and 20 µg/mL anti-IFNγ. After 5 days, RNA was harvested and analyzed by qPCR. (C) YFP^{FL/FL} or DNMA M^{FL/FL} CD4⁺ T cells were Tat-cre treated and rested for 24 hours in media containing 100 ng/mL IL-7. Naïve CD4⁺ T cells were then FACS sorted, labeled with CFSE, and stimulated as above under neutral conditions. After 3 days, cell proliferation was measured by FACS. (D) FACS sorted, Tat-cre treated, naïve CD4⁺ T cells from either YFP^{FL/FL} or DNMA M^{FL/FL} mice were stimulated as above under neutral conditions. Apoptosis and cell death were measured on day 5 by FACS using Annexin-V and DAPI staining.

Our observation that both Th1 and Th2 cell signature genes were sensitive to Notch inhibition in the same population suggested that Notch does not instruct one program over the other, but instead acts as a global regulator. This raised the possibility that Notch could influence both Th1 and Th2 cell signature genes, even in strongly polarizing conditions. In order to assay direct Notch effects, we utilized the GSI-washout assay (Weng et al., 2006). Notch targets are identified as transcripts that demonstrate GSI sensitivity under mock wash conditions and recover upon washout in the presence of cycloheximide.

Naïve CD4⁺ T cells were activated as described and cultured under strong Th1 or Th2 cell polarizing conditions. As published, both *Il4* and *Gata3-1a* behaved as direct Notch targets under Th2 cell polarizing conditions (Amsen et al., 2007; Amsen et al., 2004; Fang et al., 2007) (Figure 2-7A). Unexpectedly, both of these genes behaved as direct Notch targets under Th1 cell polarizing conditions, although the magnitude of their expression was greatly reduced, likely due to suppression by Th1 cell culture conditions (Figure 2-7B). Consistent with others (Minter et al., 2005), we identified *Tbx21* as a direct Notch target in Th1 cell conditioned cells, but surprisingly also observed that *Tbx21* behaved as a direct Notch target even under Th2 cell polarizing conditions (Figure 2-7B). To confirm the unbiased behavior of Notch under polarizing conditions, chromatin immunoprecipitation (ChIP) assays were performed. Consistent with GSI-washout results, Notch1 bound the previously reported *Il4* HS5, *Gata3-1a*, and *Tbx21* Notch1 binding sites (Figure 2-7C). The magnitude of Notch binding was similar in both Th1 and Th2 cell polarizing conditions suggesting that the polarizing conditions do not bias Notch binding to these critical Th1 and Th2 cell targets. This is also show endogenous Notch1 binds *Tbx21* in primary cells, confirming previous studies (Minter et al., 2005). Together with GSI-washout data, our ChIP findings confirm that Notch concurrently regulates both Th1 and Th2 programs, independent of cytokine signals.

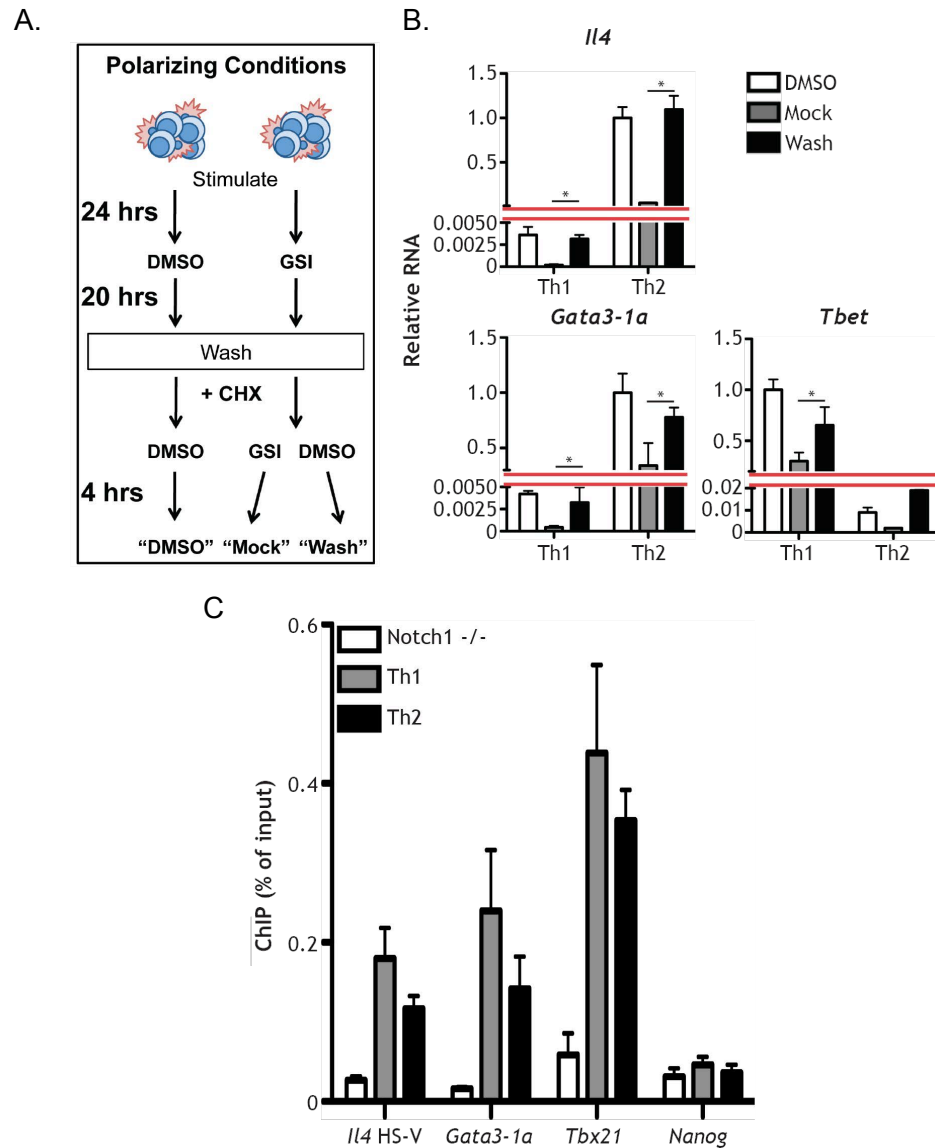


Figure 2-7: Notch concurrently regulates both the Th1 and Th2 programs. (A) Schematic of experimental design for the GSI washout assay. WT CD4⁺ T cells were stimulated as above and cultured under either Th1 or Th2 culture conditions. After 24 hours, cells were treated with either DMSO or GSI for 20 hours. Subsequently, T cells were CD4⁺ MACS purified and cells cultured in DMSO were replated in DMSO and cells cultured in GSI were either replated in GSI (Mock) or in DMSO (Washout) for 4 hours; all cells were placed in media containing CHX. (B) RNA was then harvested and analyzed by qPCR. (C) WT CD4⁺ T cells were stimulated as above. After 2 days, cells were fixed and ChIP was performed using anti-Notch1 antibody, with Nanog serving as an internal negative control. As a negative control for the assay and antibody, anti-Notch1 ChIP was performed on CD4-cre x Notch1^{FL/FL} CD4⁺ T cells. *, P < 0.05

Notch simultaneously orchestrates multiple Th cell programs by sensitizing cells to exogenous cytokine

Having observed that Notch regulates both Th1 and Th2 cell programs, we hypothesized that Notch could regulate these targets in other Th cell populations, such as Th17 cells. To test this, naïve CD4⁺ T cells were activated as described, cultured under strong Th17 cell differentiating conditions, and subjected to the GSI-washout assay. Consistent with Th1 and Th2 cell observations, *Il4* and *Tbx21* both behaved as direct Notch targets in Th17 polarized cells (Figure 2-8A); *Gata3-1a* transcript was undetectable under these conditions (data not shown). To test if Notch1 binding was conserved between Th1, Th2, and Th17 cells, ChIP analysis was performed. In keeping with our GSI washout observations, Notch1 bound to the *Il4*, *Gata3-1a*, and *Tbx21* loci in Th17 polarized cells, demonstrating that even though *Gata3-1a* was expressed at levels below the limit of detection, Notch still occupied this region. These data illustrate that the concurrent regulation of Th cell programs by Notch is not a unique feature of Th1 and Th2 cells, but rather a conserved function of Notch signaling in mature, activated CD4⁺ T cells.

To further demonstrate that Notch functions to regulate multiple cell programs from a common progenitor, we assayed whether the Th17 cell signature genes *Il17a* and *Rorc* behaved as direct Notch targets, regardless of the cytokine environment. Naïve CD4⁺ T cells were activated as described, cultured under Th1, Th2, or Th17 cell conditions, and subjected to the GSI washout assay. Although the expression varied by condition, *Il17a* and *Rorc* both behaved as direct Notch targets in all three Th cell types (Figure 2-8C).

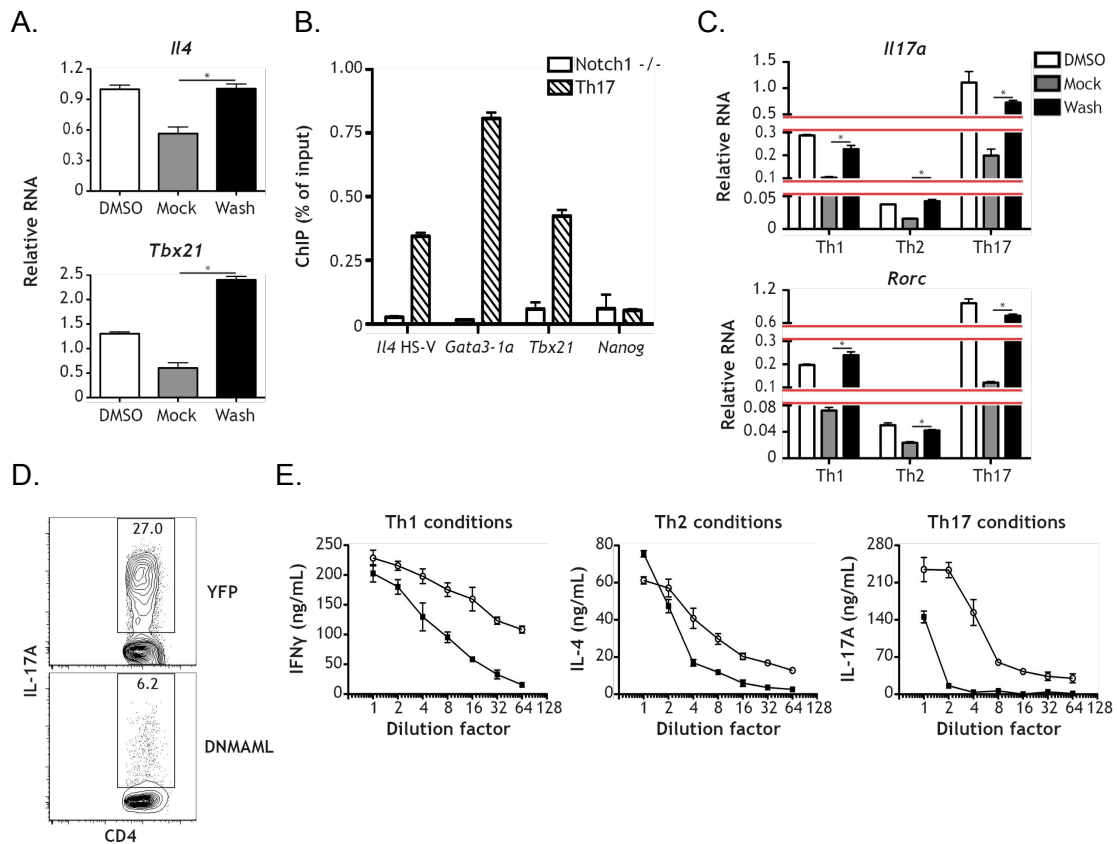


Figure 2-8: Notch simultaneously orchestrates multiple Th cell programs. WT CD4⁺ T cells were stimulated as described under Th17 culture conditions. (A) After 24 hours, cells were treated with either DMSO or GSI for 20 hours and then subjected to a GSI washout assay. Cells cultured in DMSO were replated in DMSO and cells cultured in GSI were either replated in GSI (Mock) or in DMSO (Washout) for 4 hrs. RNA was then harvested and analyzed by qPCR. (B) After 2 days, cells were fixed and ChIP was performed using anti-Notch1 antibody, with Nanog serving as an internal negative control. Anti-Notch1 ChIP was performed on CD4-cre x Notch1^{FL/FL} CD4⁺ T cells as an experimental and antibody negative control. (C) A GSI washout assay was performed as above on activated CD4⁺ T cells cultured under either Th1, Th2, or Th17 polarizing conditions. At the end of the assay (48 hrs), RNA was harvested and analyzed by qPCR. (D) Naive CD4⁺ T cells were FACS sorted from Tat-Cre treated YFP^{FL/FL} or DNMA1L^{FL/FL} CD4⁺ T cells. Cells were then stimulated as above, and cultured under Th17 conditions. After 3 days, cytokine production was measured by intracellular FACS. (E) WT CD4⁺ T cells were stimulated as described and then cultured in a titration series of two-fold serially diluted Th1, Th2, or Th17 conditioned media, in the presence of either DMSO or GSI. After 5 days, cells were restimulated and supernatants were analyzed by ELISA two days later. *, P < 0.05

To confirm that the observed effects on Th17 cell signature gene transcription were biologically significant, we expressed DNMA1 in naïve CD4⁺ T cells using Tat-Cre, as described, and activated them under Th17 differentiating conditions. We observed at day 3 that DNMA1 expression resulted in a marked reduction in the frequency of IL-17A producing cells (Figure 2-8D).

In light of reports showing that exogenous cytokine has the capacity to rescue cytokine production in Notch loss of function models (Amsen et al., 2004; Tu et al., 2005), we hypothesized that an important function of Notch was to sensitize CD4⁺ T cells to polarizing factors, especially when these factors may be limiting. To test this, CD4⁺ T cells were activated in the presence of serially diluted Th1, Th2, or Th17 polarizing cytokines, with media containing either DMSO or GSI. If Notch played a role in sensitizing cells to exogenous cytokine, we would predict that Notch inhibition would minimally affect differentiation at high levels of differentiating factors, but exert a negative effect on differentiation when exogenous cytokines were diluted. When cells were cultured in the presence of DMSO, secretion of IFN γ , IL-4, and IL-17A displayed an expected dose dependent response to polarizing cytokine, with maximal cytokine secretion observed in undiluted differentiating media (Figure 2-8E). Consistent with our hypothesis, when cells were cultured with GSI, cells displayed similar levels of cytokine secretion to DMSO control cells when differentiating cytokine was undiluted, but a much more rapid and marked decay in cytokine secretion when differentiating factors became limiting (Figure 2-8E). Altogether, these findings demonstrate that Notch simultaneously orchestrates multiple Th cell programs by sensitizing cells to exogenous differentiating factors.

Notch directly regulates IFN γ expression, independently of Tbet.

As Notch regulates a *Gata3:Il4* axis in Th2 cells and an *Il17a:Rorc* axis in Th17 cells, we hypothesized that Notch might similarly regulate a *Tbx21:Ifng* axis in Th1 cells. To confirm that *Ifng* is a direct Notch target, we performed a GSI washout assay on cells cultured under strong Th1, Th2, and Th17 polarizing conditions. Similar to *Gata3-1a*, *Il4*, and *Tbx21*, *Ifng* behaved like a direct Notch target under all three culture conditions (Figure 2-9A).

To exclude the possibility that effects of Notch on *Ifng* resulted from indirect effects on *Tbx21*, we inhibited Notch signaling in *Tbx21*^{-/-} cells. Naïve CD4⁺ T cells from WT and *Tbx21*^{-/-} mice were activated and retrovirally transduced with vector control (MigR1) or DNMAHL. To obtain sufficient IFN γ expression in Tbet deficient cells, cells were cultured in the presence of IL-12 and neutralizing anti-IFN γ and anti-IL-4 antibodies, conditions that allow Tbet-independent IFN γ production (Schulz et al., 2009; Usui et al., 2006) and exclude confounding paracrine effects between transduced and untransduced cells. Transduction of either WT or *Tbx21*^{-/-} cells with DNMAHL decreased the fraction of cells producing IFN γ as well as the total amount of IFN γ secreted (Figure 2-9B-C). Collectively, these data suggest that Notch directly regulates *Ifng* expression, independently of its role in regulating *Tbx21*, and identify *Ifng* as a Notch target.

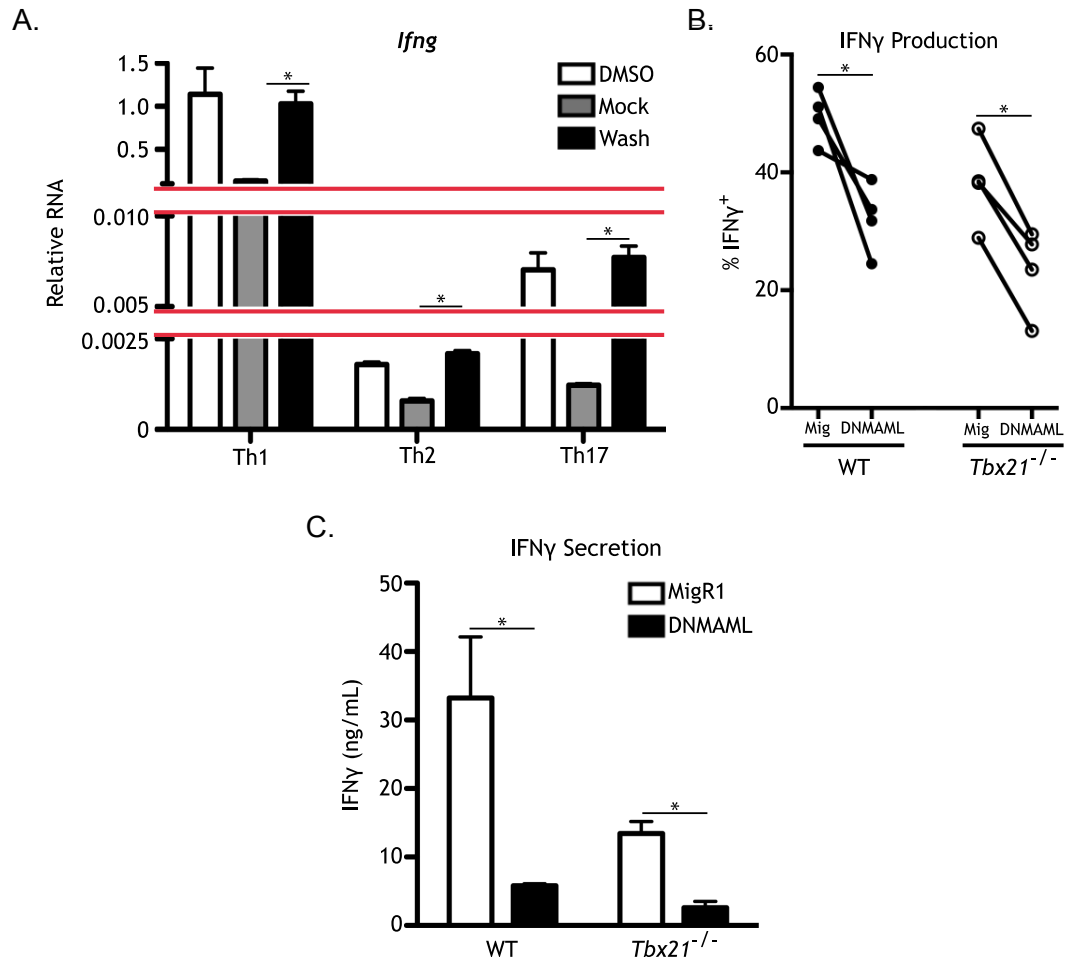


Figure 2-9: Notch directly regulates *Ifng* expression. (A) WT CD4⁺ T cells were stimulated as described under either Th1, Th2, or Th17 culture conditions. 24 hours later, cells were treated with either DMSO or GSI for 20 hours and then subjected to a GSI washout assay. Cells cultured in DMSO were replated in DMSO and cells cultured in GSI were either replated in GSI (Mock) or in DMSO (Washout) for 4 hours. RNA was then harvested and analyzed by qPCR. (B) Schematic of experimental design. WT and Tbet KO naive CD4⁺ T cells were stimulated as above and cultured with IL-12 (5 ng/mL), anti-IL-4 (20 μ g/mL), and anti-IFN γ (20 μ g/mL). After 24 hours, cells were retrovirally transduced with either vector control (Mig) or DNMAHL. (C) Cytokine production was measured by intracellular FACS and (D) cytokine secretion by ELISA, 48 hours post-transduction. *, P < 0.05

Notch1 binds to the Ifng CNS-22

To determine the mechanism by which Notch directly regulates *Ifng*, we searched for RBPJ binding sites in regions known to be critical for *Ifng* transcription (Hatton et al., 2006). Three RBPJ elements were identified in the *Ifng* CNS-22, a conserved enhancer that is required for *Ifng* expression in T and NK cells (Figure 2-10A) (Hatton et al., 2006). Primers were designed for ChIP that flanked the strongest and most conserved RBPJ binding site in the region. Two days after stimulation, CD4⁺ T cells exhibited Notch1 binding at the *Ifng* CNS-22 (Figure 2-10B). Similar to *Il4*, *Gata3-1a*, and *Tbx21* (Figure 3F), Notch1 bound the *Ifng* CNS-22 site in Th1, Th2, and Th17 cells (Figure 2-10B).

A.

| | |
|----------------|--|
| Mouse | AAAGGCAACTTCTTTCTTCTCAGGGTGATCCACAGGAAGGAGATGGGAAGTCAGATCAAA |
| Rat | ACAG-CAACTTCTTTCTTCTCAGGGTGATCCACAGGAAGGAGATGGGAAGTCAGACCAAA |
| Human | -----ATTTCTTTTCTTCTCAGGGTGACCCACAGGAAGGACATGGGAAGTTAGGTCAGA |
| Rhesus Macaque | -----ATTTCTTTTCTTCTCAGGGTGACCCACAGGAAGGACATGGGAAGTTAGGTCAGA |
| Dog | AGAGCAATTTCTTCTTCTTCTTGGTGACCCACAGGAACAGAGGGGAAGTCGGGTC-GA |
| Cow | AAAGCCATTTCTTCTTCTCAGGGTGACCCACAGGAAGGAGAGGGGAAGCCAGGTC-AA |
| Mouse | GCTGGCTTAACACCTCTGTCTTGGGAAATCCTATCAGGTGACTAGAAAGAAAACCTAAGA |
| Rat | GCTGGCTTGACACCTCTGTCTTGGGAAATCCTATCAGGTGACTAGAAA---AACCTAAGA |
| Human | ATTGGCTTGACACCTCTGTCTTGGGAAATCCTATCAGGTGACTAAGAAAGAAAAC--AACCTAAGA |
| Rhesus Macaque | ATTGGCTTGACACCTCTGTCTTGGGAAATCCTATCAGGTGACTAAGAAAGAAAACATAACC |
| Dog | AATGGCTTAACACCTCTGTCTTGGGAAATCCTATCAGGTGACTGAAAGAAAACCTTAACCT |
| Cow | CTTGACTTGACACCTCTGTCTTGGGAAATCCTATCAGGTGACTGAAAGAAAACCTTAACC |
| Mouse | GGCCAAATTTCTGCTCATTTGTCAAAGACAGGAGGAGGTTGGGGGAAGTCCCTGACCCAC |
| Rat | GGCCAAATTTCTGCTCATTTGTCAAAGACAGGAGGCAAGTTGGGGGAAGTCCCTGACCCAC |
| Human | CACCAAGTTACTGCTCTTTGCCGAGAGATGGAAGGCACCTTGGGGGAAGTCCCTGACCCAC |
| Rhesus Macaque | CACCAAGTTACTGCTCTTTGCCGAGAGATGGAAGGCACCTTGGGGGAAGTCCCTGACCCAC |
| Dog | CGCCAAATTTCTGCTCTTTGCCGAGAGATGGAAGGCACCTTGGGGGAAGTCCCTGACCCAC |
| Cow | CGCCAAATTTCTGCTCTTTGCCGAGAGATGGAAGGCACCTTGGGGGAAGTCCCTGACCCAC |

B.

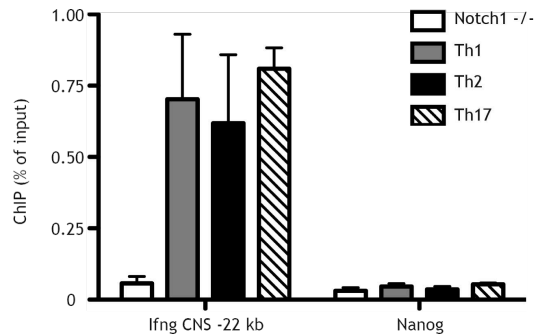


Figure 2-10: Notch binds to the *Ifng* CNS-22. (A) Multiple species alignment of the *Ifng* CNS-22 region, using NCBI DCODE.org. Highly conserved strong (red), moderate (orange), and weak (yellow) RBPJ binding sites are highlighted. Primer sequences used for ChIP are underlined. (B) WT CD4⁺ T cells were stimulated as described and cultured under Th1, Th2, or Th17 conditions. After 2 days cells were fixed and ChIP was performed using anti-Notch1 antibody, with Nanog serving as an internal negative control. Anti-Notch1 ChIP on CD4-cre x Notch1^{FL/FL} CD4⁺ T cells served as a negative control for the antibody and assay.

Notch1 and Tbet synergize to drive transcription from the Ifng CNS-22

The observation that Notch1 binds the *Ifng* CNS-22 suggested that Notch directly regulates *Ifng* expression through this element. To test this, we utilized reporter constructs for the *Ifng* CNS-22 region (Hatton et al., 2006). These constructs contain either the minimal 468 bp *Ifng* promoter (Pro^{WT}) or this promoter with the *Ifng* CNS-22 placed upstream (CNS^{WT}Pro^{WT}). Two putative T-box half-sites were identified in the *Ifng* CNS-22 fragment and mutant constructs were generated to both the upstream (CNS^{MT1}Pro^{WT}) and downstream (CNS^{MT2}Pro^{WT}) T-box half-sites (Figure 2-11A) (Hatton et al., 2006). Although the CNS^{MT2}Pro^{WT} construct was originally described as a Tbet binding site mutant, this same mutation also ablates the most highly conserved RBPJ binding site in *Ifng* CNS-22. To test if Notch was capable of driving transcription through the *Ifng* CNS-22 at this site in cells capable of endogenous IFN γ production, activated Jurkat cells were transfected with vector control (pcDNA) or the constitutively active Notch1 intracellular domain ICN1 (Aster et al., 2000), as well as a reporter containing pGL3, Pro^{WT}, CNS^{WT}Pro^{WT}, or CNS^{MT2}Pro^{WT} (Figure 2-11B). Addition of CNS^{WT}Pro^{WT} resulted in slightly increased reporter activity that was markedly enhanced upon the addition of ICN1, suggesting that Notch has the capacity to act on this enhancer element. When CNS^{MT2}Pro^{WT} was used, the increased activity observed from the CNS^{WT}Pro^{WT} was lost and the addition of ICN1 failed to enhance reporter activity (Figure 2-11B). These data suggest that Notch1 is capable of enhancing transcription through the *Ifng* CNS-22.

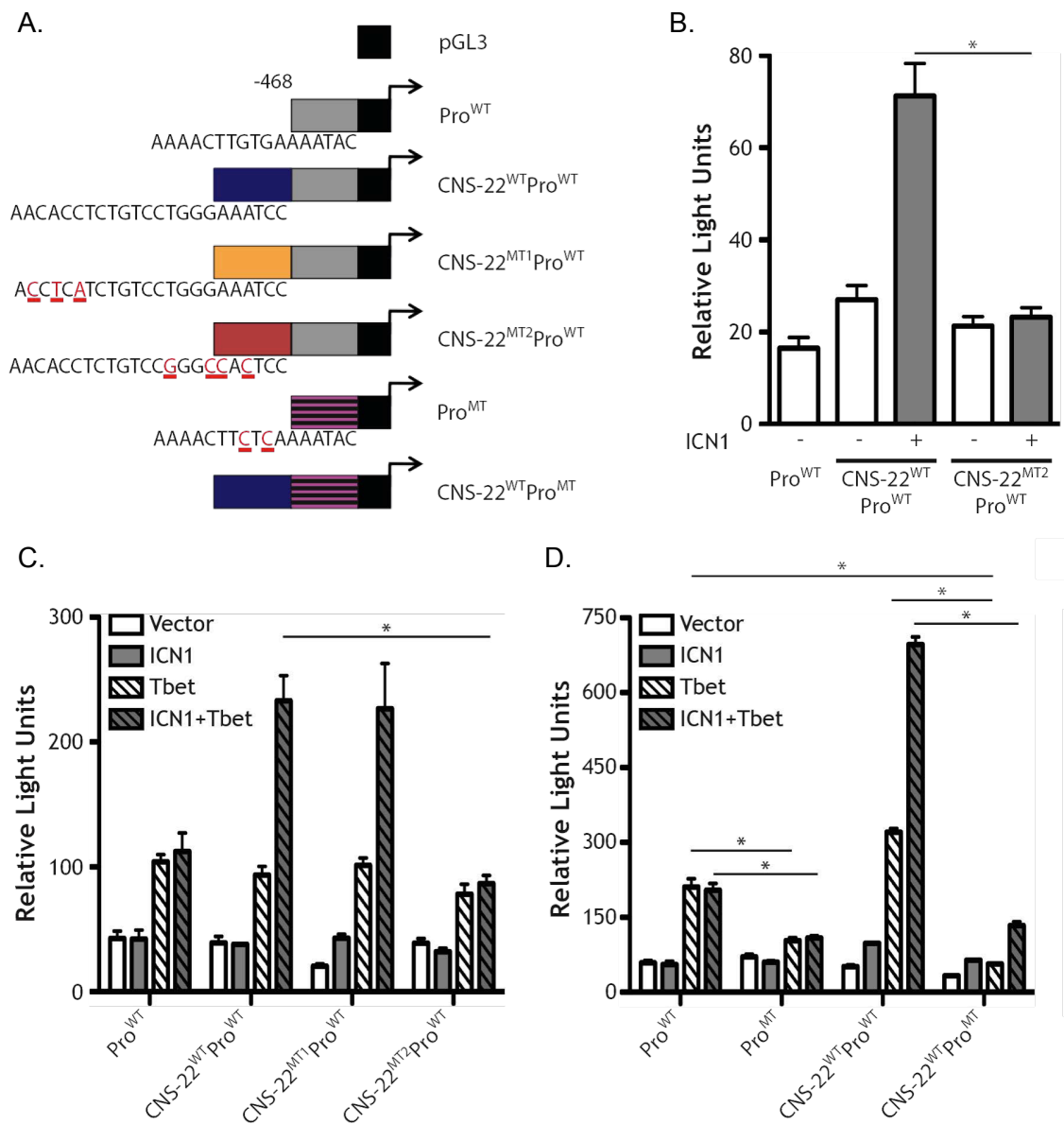


Figure 2-11: Notch1 and Tbet synergize to drive transcription from the *Ilfn* CNS-22. (A) Schematic of the luciferase constructs used, as detailed in the methods. (B) 2×10^5 Jurkat cells were transfected with renilla control (pRLTK), either the Pro^{WT}, Pro^{WT}CNS^{WT}, or Pro^{WT}CNS^{MT2} luciferase reporter constructs, and either a vector control or ICN1, using DMRIE-C liposomes. After 20 hours, cells were stimulated with PMA and ionomycin for 4 hours. Cells were then washed and luciferase activity was measured. (C) 2×10^4 U2OS cells were transfected with pRLTK, either the promoter Pro^{WT}, Pro^{WT}CNS^{WT}, Pro^{WT}CNS^{MT1}, or Pro^{WT}CNS^{MT2} reporter constructs, ICN1 or vector control, and Tbet or vector control. After 48 hours, luciferase activity was measured. (D) 2×10^4 U2OS cells were transfected with pRLTK, either the Pro^{WT}, Pro^{MT}, Pro^{WT}CNS^{WT}, or Pro^{MT}CNS^{WT} reporter constructs, ICN1 or vector control, and Tbet or vector control. After 48 hrs, luciferase activity was measured. *, $P < 0.05$

Although the Jurkat cell data established that Notch1 can drive transcription from the *Ifng* CNS-22 in a mature T cell line, it remained possible that Notch1 indirectly enhanced luciferase activity by promoting *Tbx21* transcription. In addition, the loss of luciferase activity observed when CNS^{MT2}Pro^{WT} was expressed may have resulted from a loss of Tbet binding, rather than the loss of RBPJ binding at the same site. To test these alternate hypotheses, we utilized U2OS cells, which do not express endogenous *Tbx21* and express very low amounts of Notch1 (data not shown). U2OS cells were transfected with the reporter constructs described above as well as CNS^{MT1}Pro^{WT}, which ablates the upstream T-box half-site without disrupting the RBPJ binding site. Tbet overexpression in U2OS cells increased luciferase activity in all constructs (Figure 2-11C). This effect appeared primarily due to Tbet activity on the *Ifng* minimal promoter, as the addition of the *Ifng* CNS-22 and mutation of either T-box half-site had no additional effect on reporter activity (Figure 2-11C).

In contrast to Jurkat cells, ICN1 did not increase luciferase activity in U2OS cells, suggesting that other factors endogenous to Jurkat cells, such as Tbet, may be required to cooperate with Notch1 activity. Accordingly, co-expression of Tbet and ICN1 synergistically increased, luciferase activity from CNS^{WT}Pro^{WT}. Moreover, the enhanced luciferase activity was specific to CNS^{WT}Pro^{WT} and was not observed with Pro^{WT}, suggesting that the synergy between Notch1 and Tbet resulted from Notch1 activity at the *Ifng* CNS-22 (Figure 2-11C).

To directly address this possibility, we utilized the construct in which the upstream T-box half-site was mutated (CNS^{MT1}Pro^{WT}). In cells expressing this reporter, luciferase activity

was similar to the $\text{CNS}^{\text{WT}}\text{Pro}^{\text{WT}}$ under all conditions, suggesting that the primary effect of Tbet was through its activity on the promoter. In contrast, when the conserved RBPJ binding site was mutated ($\text{CNS}^{\text{MT2}}\text{Pro}^{\text{WT}}$), luciferase activity was comparable to the Pro^{WT} construct (Figure 2-11C). Collectively, these data suggest that ICN1 acts at the *Ifng* CNS-22 enhancer and Tbet acts at the *Ifng* promoter.

To directly assay the site of Tbet activity, we mutated the T-box site in the *Ifng* promoter leaving the putative Tbox sites in the CNS-22 intact (Tong et al., 2005) (Figure 2-11A). When U2OS cells were transfected with Pro^{MT} , neither Tbet alone nor Tbet plus ICN increased reporter activity to that observed in Pro^{WT} (Figure 2-11D). Moreover, mutation of the promoter in the context of CNS-22 ($\text{CNS}^{\text{WT}}\text{Pro}^{\text{MT}}$) ablated the ability of either Tbet alone or Tbet plus ICN1 to increase reporter activity (Figure 2-11D). Overall, these data demonstrate that binding of Notch at the *Ifng* CNS-22 is insufficient to activate transcription by itself and that Tbet binding at the promoter leads to weak activation; however, the combination of Notch binding to the CNS-22 and Tbet binding to the *Ifng* promoter leads to a synergistic increase in *Ifng* transcription.

Discussion

Within the lymphoid compartment, Notch is understood to selectively promote one lineage outcome at the expense of alternate fates (Radtke et al., 2010). This instructive paradigm was proposed to explain Th cell differentiation, however, the emerging data are difficult to reconcile with this model as Notch promotes mutually exclusive cell fates from a multipotential cell (Amsen et al., 2004; Maekawa et al., 2003; Minter et al., 2005; Mukherjee et al., 2009; Tu et al., 2005). Here, we present data demonstrating that Notch acts as an unbiased amplifier of the Th1, Th2, and Th17 cell programs by sensitizing cells to environmental signals.

By acutely inhibiting Notch signaling under neutralizing conditions, we reveal a role for Notch in simultaneously orchestrating both Th1 and Th2 programs. GSI treatment and acute DNMA1 expression synchronously reduced IL-4, IFN γ , and Tbet protein and mRNA for *Il4*, *Ifng*, *Tbx21*, and *Gata3-1a*, demonstrating that Notch lacks selectivity in regulating critical Th cell program targets. We further observed that Notch regulates Th17 target genes even when their expression is suppressed under Th1 and Th2 conditions and *vice versa*. Although the polarizing conditions influence the magnitude of gene expression, the ability of Notch to bind these key loci was unchanged, illustrating that the cytokine environment does not impact the ability of Notch to regulate its targets. Accordingly, our work suggests that Notch plays a critical role in reinforcing Th cell differentiation at physiologic levels of cytokine signaling, which would be important early during immune responses when differentiating cytokine cues are limiting. In addition, these findings help reconcile conflicting reports in the literature that emphasized the ability of Notch to preferentially regulate specific Th programs.

In related work, Ong et al. contended that Notch signaling itself had minimal impact on Th differentiation, but rather upstream cytokine signals directed Notch to selectively enhance individual Th responses (Ong et al., 2008). Consistent with our findings, these studies argued that Notch lacks instructive capacity. Our *T. muris* studies provide the first *in vivo* loss-of-function data confirming that Notch is not required for instruction, however our GSI-washout and ChIP data provide a distinct mechanistic view of Notch function during Th differentiation. Rather than requiring cytokine signals to condition Notch selectivity, we find that Notch binds and regulates target loci without regard to cytokine signals. Moreover, we further show that Notch concurrently regulates Th cell programs even under neutralizing conditions. Thus, our work suggests that the activity of Notch is not dictated by cytokine signaling, but rather that Notch simultaneously facilitates transcription of multiple programs regardless of polarizing cues.

In addition to providing a unifying model for Notch in Th cell differentiation, we present definitive genetic loss-of-function and molecular data evincing a role for Notch in the Th1 program. Not only do our data demonstrate for the first time that endogenous Notch1 binds *Tbx21* and that *Tbx21* is a direct Notch target in primary CD4⁺ T cells, but we also show that *Ifng* is a novel, direct Notch target, independent of Notch's role in regulating *Tbx21*. While the Notch effects on *Ifng* are independent of its activity on *Tbx21*, Notch does not appear capable of driving *Ifng* transcription by itself, consistent with a model in which Notch lacks the capacity to instruct Th differentiation. Both factors are needed for optimal *Ifng* expression, where they bind different regulatory elements. Moreover, the *Tbx21*^{-/-} studies suggest that Notch is capable of collaborating with factors other than Tbet, as DNMA1L suppressed *Ifng* expression in its absence. The original report using these luciferase constructs found that Tbet overexpression was capable of enhancing

luciferase activity of the WT CNS-22 construct when cells were stimulated with PMA and ionomycin (Hatton et al., 2006), which also induces NFκB binding to the *Ifng* CNS-22 (Balasubramani et al., 2010). As the *Ifng* CNS-22 contains multiple regulatory motifs, these data suggest that Tbet activity at the *Ifng* promoter synergizes with multiple factors regulating the *Ifng* CNS-22, including Notch and NFκB. Altogether, these data illustrate the dynamism of the *Ifng* promoter and CNS-22 and demonstrate how these elements have the potential to integrate inputs from multiple pathways.

The results of our reporter assays are reminiscent of the synergy between Gata3 and Notch1 at the *Il4* locus and suggest that Notch and cytokine signaling collaborate in both Th1 and Th2 differentiation (Fang et al., 2007). Furthermore, Notch appears to participate in a feed-forward loop, promoting *Tbx21* transcription and in turn synergizing with Tbet protein to enhance *Ifng* transcription. Work by Flavell and colleagues suggests that this Th1 feed-forward loop must be stabilized by other factors (Amsen et al., 2007). In their study, constitutive Notch signaling was insufficient to enforce Th1 differentiation in the presence of endogenous Gata3, demonstrating that low levels of Gata3 act as a failsafe against runaway Th1 differentiation in response to Notch activation. In addition to transcriptional regulation, Notch can regulate IFNγ secretion in an RBPJ-independent manner, suggesting that Notch regulates the CD4⁺ T cell IFNγ response at multiple, mechanistically distinct levels (Auderset et al., 2012). Additionally, a recent human T cell study implicated a role for Notch in Th1 differentiation (Le Friec et al., 2012). Together, these data firmly establish Notch as a key regulator of the Th1 program.

As well as revealing a definitive role for Notch in promoting Th1 differentiation, our data further clarify the mechanism by which Notch regulates Th2 differentiation. The findings from anti-IFNγ mAb treated *T. muris* infected CCD mice indicate that the role of Notch

during *in vivo* Th2 inflammation is similar to what has been reported for NFκB2, IL-25, and TSLP (Artis et al., 2002; Owyang et al., 2006; Taylor et al., 2009). Like Notch, mice deficient for each of these factors displayed susceptibility to helminth infection and impaired Th2 immune responses, however blockade of Th1 responses in these mouse models resulted in restoration of the Th2 response and worm expulsion. Moreover, unlike the role for Notch in regulating Th1 differentiation, we observe a major role for Notch in promoting IL-4 production but minimal impact on total Gata3, suggesting that additional factors, such the Notch target Tcf1, may be required to fully engage the Th2 program (Yu et al., 2009). These studies collectively illustrate a clear distinction between Th2 initiating factors, such as NFκB1 (Artis et al., 2002), and the multiple inputs that maintain an optimal Th2 response *in vivo*, but are dispensable for Th2 program initiation, such as Notch.

In addition to regulating Notch-dependent IL-4 production in Th2 cells, recent work demonstrated that the IL-4 HS5 region is critical for T follicular helper cell (Tfh) production of IL-4 (Harada et al., 2012; Vijayanand et al., 2012). These findings raise the possibility that Notch inhibition in the *T. muris* studies may impact both Th2 and Tfh subsets during infection and the combined effects on these two populations contribute to the phenotype observed. Importantly, anti-IFNγ treatment restored productive immunity against helminth infection; and therefore, the role that Notch signaling plays in Tfh biology is either redundant with its function in Th2 cells or not essential for Tfh differentiation, similar to what we observe for Th1, Th2, and Th17 subsets.

With the recent recognition of Th cell plasticity, particularly at early time points, our data suggest that Notch functions to ensure that activated CD4⁺ T cells overcome a Th cell commitment threshold (Murphy and Stockinger, 2010). In this manner, Notch tunes the

responsiveness of an activated CD4⁺ T cell to a specific Th cell program by sensitizing cells to limiting environmental differentiation cues. Thus, depending on the inflammatory environment for a given immune response, the requirement for Notch will vary depending on whether the strength of the differentiating signals a T cell receives are sufficient to achieve a signaling threshold for Th cell commitment. For example, during *T. muris* infection, our model argues that Notch sensitizes T cells to limiting Th2 differentiating cues, while residual IFN γ signals destabilize the Th2 circuitry when Notch signaling is abrogated. This model would also explain why Th1 differentiation occurs independently of Notch during *Leishmania* infection, as we would predict that the environmental differentiating signals are sufficient to overcome a commitment threshold (Amsen et al., 2004; Tu et al., 2005). Alternatively, in the context of GvHD, which is characterized by a mixed Th cell response, Notch is required to sensitize cells to subthreshold signals and achieve optimal IFN γ production (Zhang et al., 2011). While our data favor a model in which Notch regulates Th differentiation by sensitizing cells to their environment, Notch likely plays additional roles in other aspects of Th biology, such as survival and metabolism, that warrant future study.

Overall, these findings offer a paradigm for Notch in the immune system. In addition to its roles as an arbiter of alternate fate decisions and a key regulator of cell survival, proliferation, and metabolism, we reveal that Notch also acts to potentiate multiple fates from a single progenitor. Not only does this paradigm reconcile previously conflicting studies, but it also suggests that manipulating the amounts of Notch signaling in Th cell mediated pathologies may have therapeutic benefit.

CHAPTER 3: Notch signaling lacks instructional or oncogenic activity post- β -selection

Abstract

Notch signaling is classically understood to instruct cell fates during development and to drive oncogenesis when hyperactivated during hematopoiesis. Enforced activation of Notch signaling in bone marrow progenitors or T lineage precursors prior to the β -selection checkpoint instructs T lineage development and drives T cell acute lymphoblastic leukemia. Here we find that activation of Notch signaling after β -selection fails to instruct cell fates and does not promote leukemia. Instead, mice develop a lethal autoinflammatory disorder. This disease results from the ability of Notch signaling to concurrently promote Th1, Th2, and Th17 cell differentiation. Moreover, this study reveals a novel role for Notch signaling in destabilizing Treg differentiation. Notch signaling acts to desensitize T cells to TGF β , such that inhibiting Notch signaling promotes Treg differentiation in non-permissive conditions. Notch's dual role in promoting inflammatory Th cell differentiation and destabilizing Treg differentiation makes it a key regulator of the Th9-Treg and Th17-Treg axes and an attractive therapeutic target for inflammatory disorders. Altogether these findings highlight a loss in oncogenic potential for Notch signaling after β -selection and evince a non-instructional paradigm for Notch signaling in late-stage T lineage development .

Introduction

Chapter 2 presented work showing that the Notch pathway has the capacity to sensitize activated CD4⁺ T cells, in an unbiased fashion, to environmental differentiation signals that promote inflammatory Th cell programs (Bailis et al., 2013). In this manner, Notch signaling is required for Th cell mediated inflammation in the context of diseases that are characterized by mixed-cytokine responses, such as *Trichurus muris* infection or graft-versus-host disease, but is not essential for diseases characterized by the efficient and dominant differentiation of a single Th cell response, such as *Listeria major* (Tu et al., 2005; Zhang et al., 2011). Unlike obligate factors, such as cytokines and co-stimulatory receptors, that have the capacity to instruct a given program and/or activate CD4⁺ T cells, these findings place Notch receptors in a unique category of auxiliary factors that act to boost local activating and differentiating cues in a nondiscriminatory manner.

In contrast to its unbiased role in Th cell differentiation, Notch signaling is classically understood to play an essential function in instructing cell fate decisions (Radtke et al., 2013). Within the hematopoietic compartment, Notch signaling is best known for its role in instructing T lineage development at the expense of B cells. To this end, enforced activation of the Notch pathway has been shown to potently drive T lineage commitment when the constitutively active intracellular Notch (ICN) domain is expressed in hematopoietic and early-T progenitors (Radtke et al., 2013). Moreover, high levels of sustained Notch signaling drive an oncogenic program that leads to the onset of T cell acute lymphoblastic leukemia (T-ALL) (Aster et al., 2011). In the peripheral T cell compartment, expression of ICN has been reported to selectively drive Th1 and Th2 cell differentiation (Amsen et al., 2007; Fang et al., 2007; Maekawa et al., 2003; Minter et al.,

2005). Thus it remains possible that the Notch pathway behaves instructionally and/or signaling outcome is dictated by signal dose: such that at limiting concentrations, Notch acts in an unbiased manner, whereas above a certain threshold, the Notch pathway displays its classical instructional activity.

In order to substantiate our loss-of-function *in vitro* findings and test whether the outcome of Notch signaling is merely dictated by signal dose, we bred CD4-Cre mice to mice homozygous for a flox-stopped ICN allele (ICN^{F/F}), such that the Notch pathway is constitutively active in all T-lineage cells from the double positive (DP) stage of thymocyte development onward. All previous gain-of-function Notch studies have been performed using bone marrow progenitors or conditional models that induce Notch activation before the β -selection checkpoint of thymocyte development. In these models, activation of the Notch pathway instructs T cell development and drives T-ALL onset (Allman et al., 2001; Bellavia et al., 2000; Bellavia et al., 2002; Deftos et al., 2000; Pear et al., 1996; Pui et al., 1999; Robey et al., 1996). If Notch signal dose determines whether the Notch pathway behaves instructionally, then expression of ICN in DP thymocytes should lead to either T-ALL onset, the selective induction of a single Th cell program, or both. If Notch signaling acts by fundamentally distinct mechanisms in early and late T-lineage development, then the unbiased activity of Notch observed in our loss-of-function studies should manifest itself in these mice.

Unlike previously reported gain-of-function Notch signaling models in early T-lineage progenitors, activation of the Notch pathway after β -selection does not lead to T-ALL onset. In contrast, CD4-Cre x ICN^{F/F} (NIC) mice succumb to a lethal autoinflammatory disorder, mediated by CD4⁺ T cells and characterized by skin necrosis, multi-organ

inflammation, and wasting. Rather than promoting the differentiation of a single Th cell program, persistent Notch signaling simultaneously promotes the differentiation of Th1, Th2, and Th17 cells, in keeping with loss-of-function findings. Furthermore, these studies reveal a novel role for Notch signaling in destabilizing regulatory T (Treg) cell differentiation by desensitizing activated CD4⁺ T cells to TGFβ-derived signals, making Notch a key regulator of the Th17-Treg and Th9-Treg axes and an attractive therapeutic target for diseases resulting from CD4⁺ T cell-mediated inflammation. Altogether, these findings support a model in which the Notch pathway exerts distinct mechanistic functions in early and late in T cell development.

Results

Enforced Notch signaling post- β -selection induces a lethal autoinflammatory disorder, while failing to drive T-ALL

To test the consequences of constitutive Notch signaling in post β -selection T cells, we bred CD4-Cre mice to ICN1^{FL/FL} mice (Figure 1A), which maintains Notch signaling in T cells after β -selection. In contrast to models in which ICN1 is expressed before β -selection, CD4--ICN1 mice fail to develop T-ALL. Instead, these mice die by 200 days of age from a disease process characterized by a severe systemic autoinflammatory disorder (Figure 3-1B). CD4-cre x ICN1 mice display severe skin necrosis, colitis, and cachexia (Figures 3-1C-D). Additionally, these mice exhibit hepatosplenomegaly and small thymi (Figures 3-1E-F). Histopathologic analysis revealed extensive granulocytic infiltrates in the colon, liver, spleen, lymph nodes, and bone marrow (Figure 3-2A). Furthermore, these granulocytic infiltrates disrupted the normal tissue architecture (Figures 3-2B-C). Altogether, these data suggest that persistent Notch signaling post- β -selection induces a lethal, autoinflammatory disorder.

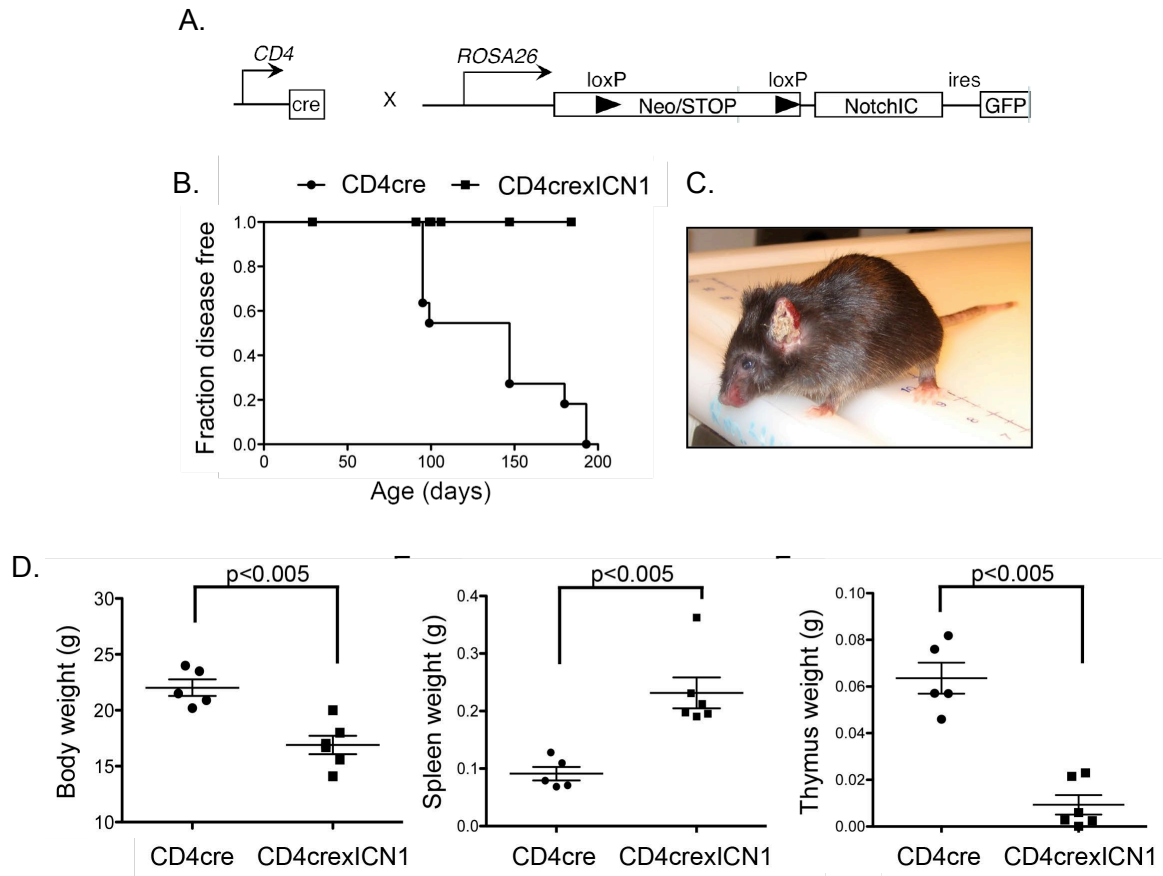


Figure 3-1: CD4-Cre x ICN1^{FL/FL} mice develop a lethal autoinflammatory disease. (A) Schematic of transgenic alleles for CD4-Cre and ICN^{FL/FL} mice. (B) Kaplan-Myer survival curve of CD4-Cre and CD4-Cre x ICN^{FL/FL} mice. (C) Representative picture of CD4-Cre x ICN^{FL/FL} mice. (D) Body weight, (E) spleen weight, and (F) thymus weight of CD4-Cre and CD4-Cre x ICN^{FL/FL} mice.

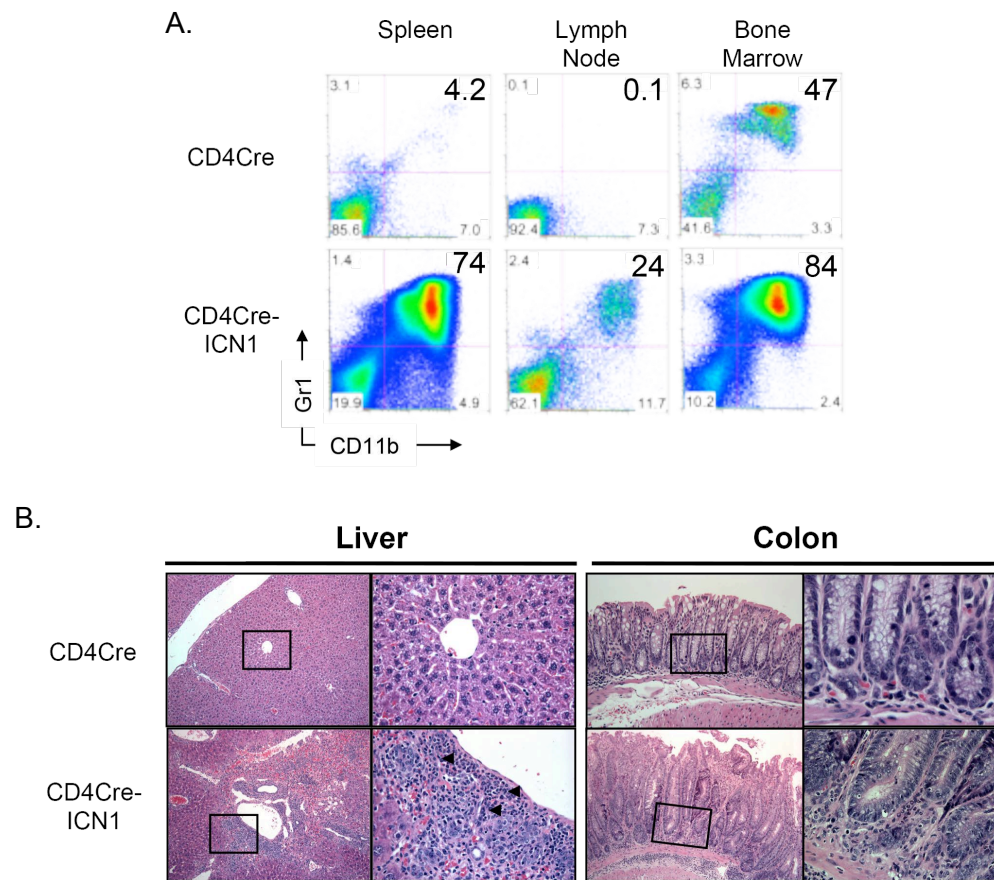


Figure 3-2: CD4-Cre x ICN1^{FL/FL} mice display severe neutrophilia and granulocyte infiltration (A) Flow cytometry of neutrophils in spleen, lymph node, and bone marrow of CD4-Cre and CD4-Cre x ICN1^{FL/FL} mice. (B) Histology of liver and colon sections from CD4-Cre and CD4-Cre x ICN1^{FL/FL} mice with H&E staining.

Helper T cells are responsible for mediating the autoinflammation observed in CD4-Cre x ICN1 mice

Although CD4-Cre activates Notch signaling in post β -selection T cells, it remained possible that the disease observed originated from indirect effects on non-T lineage populations. To confirm that the autoinflammatory phenotype was due to Notch signaling within the T cell compartment, 1×10^6 purified T cells or T-depleted splenocytes from either CD4-Cre x YFP^{FL/FL} or ICN1 mice were adoptively transferred into WT mice. Granulocyte frequency was tracked as a biomarker for disease and three weeks post-transplant, we assessed neutrophil content in the spleens of recipient mice. Even though the adoptively transferred cells were only a small fraction of circulating leukocytes in the WT recipient mice, T cells from CD4-Cre x ICN1 mice were sufficient to induce increased neutrophil recruitment, whereas CD4-Cre x YFP^{FL/FL} T cells and non-T cells from either group failed to affect granulocyte frequency (Figure 3-3A).

To further resolve the population of cells responsible for mediating the autoinflammation observed in CD4-Cre x ICN1 mice, we tested the ability of CD4⁺ and CD8⁺ T cell subsets to transfer disease. CD4⁺ or CD8⁺ T cells were FACS sorted from either CD4-Cre x YFP^{FL/FL} or CD4-Cre x ICN1 mice and 1×10^6 cells were transferred to WT mice. After four weeks, granulocyte frequency was assessed in the spleens of recipient mice. While CD8⁺ T cells from both groups and CD4⁺ T cells from CD4-Cre x YFP^{FL/FL} mice displayed normal levels of splenic granulocytes, CD4⁺ T cells from ICN1 mice were sufficient to induce granulocyte accumulation (Figure 3-3B). These data suggest that Th cells from CD4-Cre x ICN1 mice are the primary driver of disease.

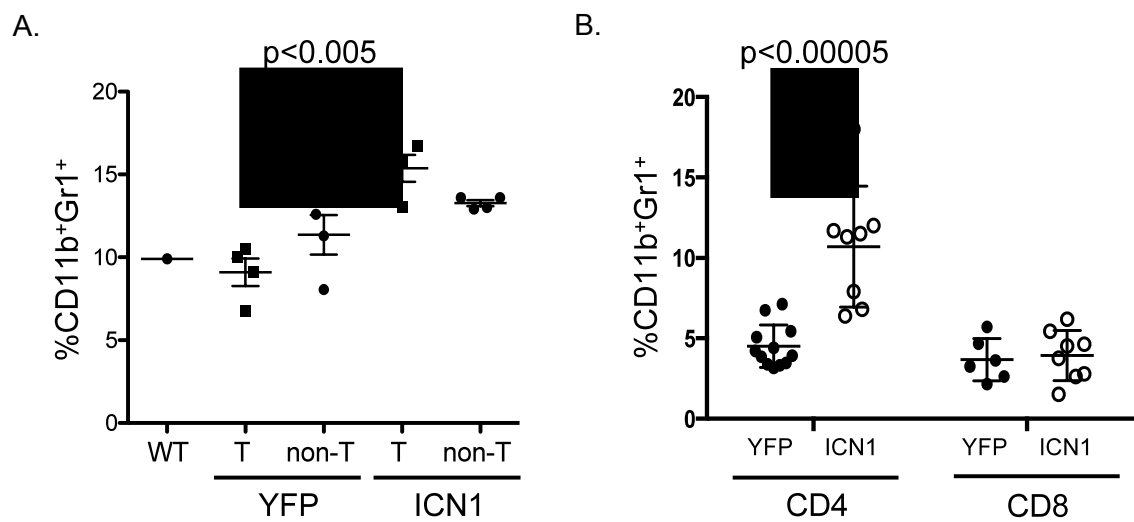


Figure 3-3: CD4⁺ T cells mediate disease in CD4-Cre x ICN1^{FL/FL} mice. (A) T cells or T-depleted cells from CD4-Cre x YFP^{FL/FL} or CD4-Cre x ICN1^{FL/FL} mice were adoptively transferred into WT mice. Neutrophil frequency in the spleens of recipient mice was analyzed by flow cytometry, three weeks post transfer. B6 mice were used as a normal control. (B) CD4⁺ and CD8⁺ T cells from CD4-Cre x YFP^{FL/FL} or CD4-Cre x ICN1^{FL/FL} were adoptively transferred to WT mice. Neutrophil frequency in the spleens of recipient mice was analyzed by flow cytometry, four weeks post transfer.

Enforced Notch expression in peripheral T cells concurrently promotes Th1, Th2, and Th17 differentiation

Having identified ICN1-expressing Th cells as the principal disease mediator, we sought to characterize the cytokine response of CD4-Cre x ICN1 CD4⁺ T cells. In contrast to previous reports that constitutive Notch1 signaling in hematopoietic progenitors or CD4⁺ T cells selectively instructs a single differentiation program (Amsen et al., 2007; Fang et al., 2007; Maekawa et al., 2003; Minter et al., 2005), our genetic model of enforced Notch signaling increased Th1, Th2, and Th17 differentiation (Figure 3A-B). *Ex vivo* stimulation of CD4-Cre x YFP^{FL/FL} or CD4-Cre x ICN1 T cells with PMA and ionomycin revealed that CD4-Cre x ICN1 Th cells increased IL-4, IFN γ , and IL-17A production. Moreover, CD4-Cre x ICN1 Th cells spontaneously produced all three cytokines in the absence of stimulation, in contrast to control cells, indicating that Notch signaling concurrently promotes multiple inflammatory Th cell programs, findings that are consistent with loss-of-function findings (Bailis et al., 2013),.

To verify that the increase in signature cytokine production of all three inflammatory Th subsets reflected *bona fide* Th cell differentiation and not an unstable, poly-activated state, we attempted to re-differentiate CD4-Cre x ICN1 T cells *in vitro* under Th1 or Th2 conditions. If hyperactivation of Notch signaling resulted in a broad increase in cytokine production by T cells, but not true Th cell differentiation, then it should be possible to differentiate these cells to a single program under stringent Th1 or Th2 conditions. Additionally, if Notch signaling has any instructional bias toward a single program, it should be revealed under these robust differentiating conditions. That is, if Notch has

instructional bias toward Th2 differentiation we should see synergy under Th2 conditions and retained IL-4 production under Th1 conditions, but not visa-versa. As expected, control T cells displayed a mixture of IL-4 and IFN γ production under non-differentiating Th0 conditions, only IFN γ production under Th1 conditions, and only IL-4 production under Th2 conditions (Figure 3C). In contrast, CD4-Cre x ICN1T cells display an increased capacity to produce both IL-4 and IFN γ under all three conditions (Figure 3C). Not only do CD4-Cre x ICN1 T cells retain IL-4 production under Th1 conditions and IFN γ under Th2 conditions, indicating that these are bona fide Th1 and Th2 differentiated cells, but these cells increased the IL-4, IFN γ double-producing population regardless of conditions, further supporting a role for Notch as an unbiased amplifier of multiple Th cell programs (Figure 3C).

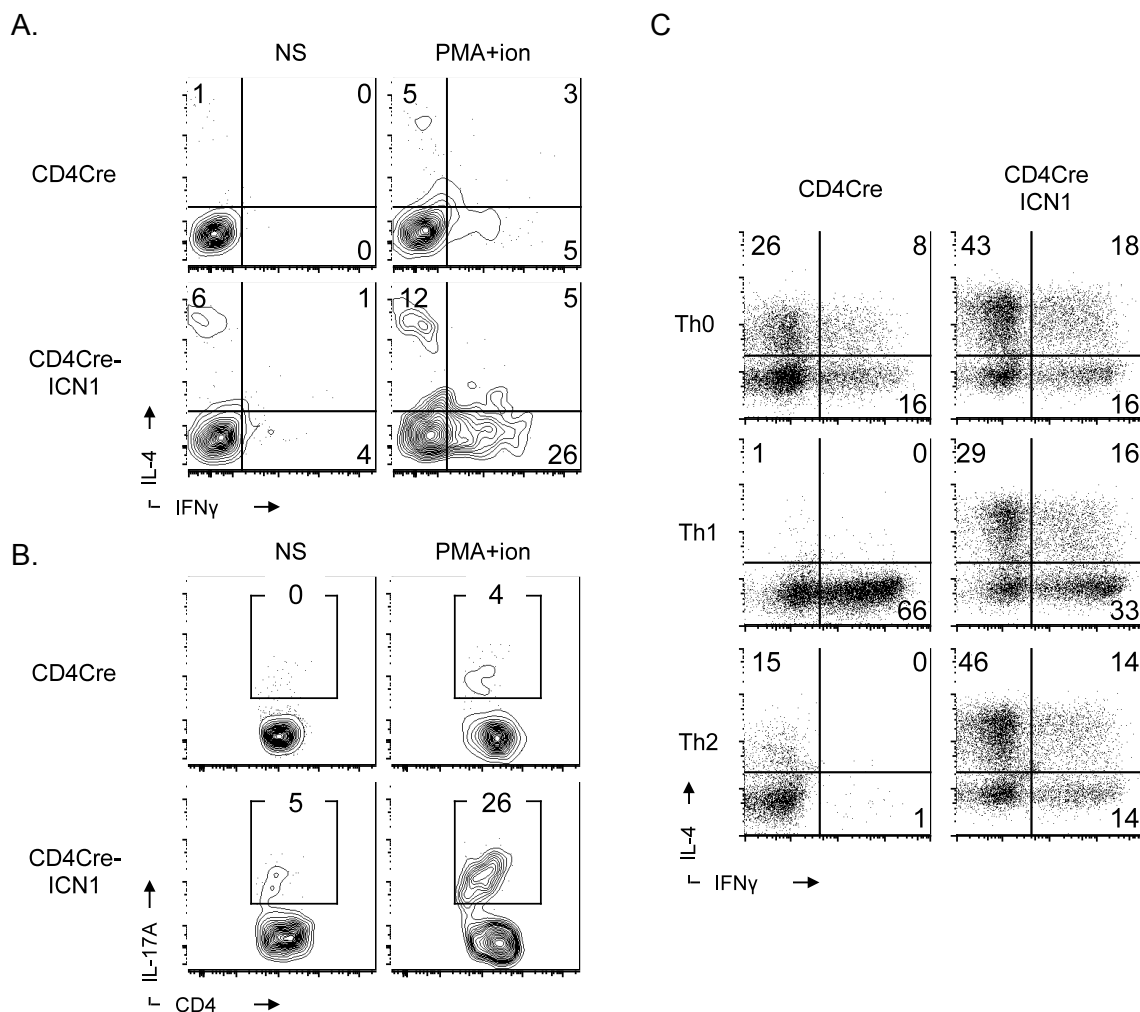


Figure 3-4: CD4⁺ T cells from CD4-Cre x ICN^{FL/FL} mice display spontaneous production of IL-4, IFN γ , and IL-17A. Lymph node cells from CD4-Cre or CD4-Cre x ICN^{FL/FL} mice were either left unstimulated or activated with PMA and ionomycin for 5 hrs and then analyzed by intracellular flow cytometry for (A) IL-4 and IFN γ or (B) IL-17A. (C) CD4⁺ T cells from CD4-Cre or CD4-Cre x ICN^{FL/FL} mice were stimulated with irradiated splenocytes, anti-CD3 ϵ , and anti-CD28, and cultured under Th0, Th1, or Th2 conditions. After 5 days, cells were restimulated with PMA and ionomycin and analyzed by intracellular flow cytometry for IL-4 and IFN γ production.

Notch signaling potentiates the differentiation of multiple inflammatory Th cell subsets in a cell-autonomous manner

While our adoptive transfer studies indicate that small numbers of CD4-Cre x ICN1 T cells are sufficient to transfer hallmarks of the disease observed in CD4-Cre x ICN1 mice to WT recipients, it remained possible that the hyper-activation of these Th cells was secondary to Notch activation in another cell type or due to the absence of a normal T cell population. To address this, bone marrow cells from either SJL controls, CD4-Cre x ICN1 mice, or a 1:1 mix from SJL and NIC mice were transferred into lethally irradiated RAG2^{-/-} recipients. In contrast to SJL bone marrow recipients that were grossly normal, both CD4-Cre x ICN1 and SJL/NIC mix recipients developed disease. CD4-Cre x ICN1 bone marrow chimeras exhibited weight loss, thymic involution, and neutrophilia, with SJL/NIC mixed chimeras showing comparable thymic atrophy and elevated granulocyte numbers and a trend toward a decrease in weight (Figure 3-5A-D).

As both CD4-Cre x ICN1 and SJL/ CD4-Cre x ICN1 bone marrow chimeras developed disease similar to that observed in CD4-Cre x ICN1 mice, we next tested whether ICN1 expression in CD4⁺ T cells from these bone marrow chimeras also promoted the differentiation of multiple Th cell subsets. Splenocytes from SJL, CD4-Cre x ICN1, and SJL/ CD4-Cre x ICN1 mixed bone marrow chimeras were stimulated *ex vivo* with PMA and ionomycin and cytokine production was assessed by intracellular flow cytometry. Restimulated SJL CD4⁺ T cells demonstrated the capacity to produce both IFN γ and IL-2, but minimal capacity to produce IL-4 or IL-17A (Figure 3-5E). SJL, GFP⁻CD4⁺ T cells from SJL/ CD4-Cre x ICN1 mixed chimeras also displayed IFN γ and IL-2 production and

a limited capacity to produce IL-17A, but showed an increased capacity to produce IL-4, compared to SJL single chimeric CD4⁺ T cells, suggesting that the presence of CD4-Cre x ICN1 T cells in the mixed chimera culture affected cytokine production by SJL cells in *trans* (Figure 3-5E). Unlike SJL cells from either culture, GFP⁺CD4⁺ T cells from both CD4-Cre x ICN1 single chimeras or SJL/ CD4-Cre x ICN1 mixed chimeras displayed an increased capacity to produce all four cytokines, including the emergence of IL-4⁺IFN γ ⁺ and IL-17A⁺IL-2⁺ multi-cytokine producing populations, consistent with a role for Notch in concurrently promoting multiple Th cell programs (Figure 3-5E). Altogether, these data indicate that the disease observed in CD4-Cre x ICN1 mice is T cell autonomous and that the CD4-Cre x ICN1 CD4⁺ T cell poly-differentiated phenotype cannot be promoted in *trans*.

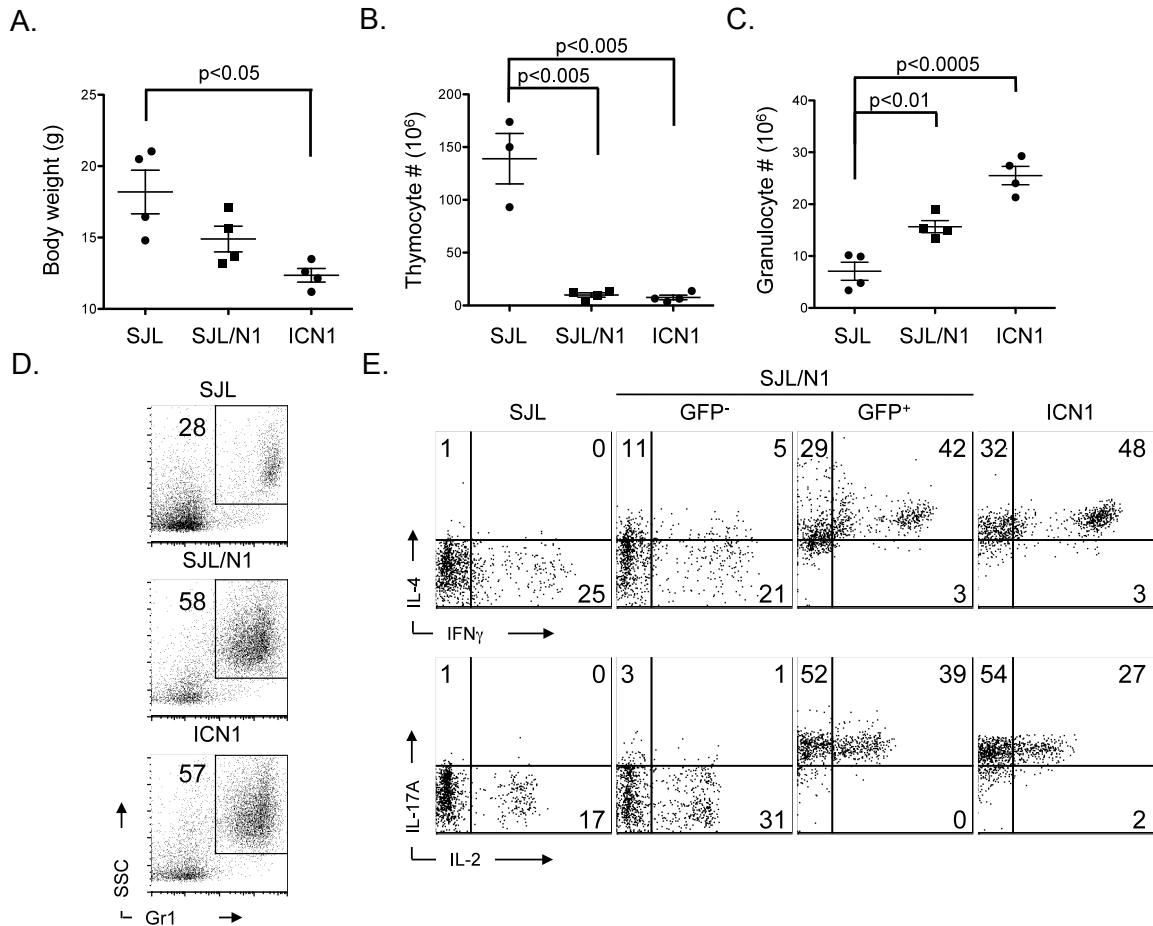


Figure 3-5: T cell defects in CD4-Cre x ICN^{FL/FL} are cell autonomous. Bone marrow cells from SJL, CD4-Cre x ICN^{FL/FL}, or both SJL and CD4-Cre x ICN^{FL/FL} mice were adoptively transferred into lethally irradiated SJL recipients. After 6 weeks, mice were analyzed. (A) Body weight of recipient mice. (B) Total thymocytes number in recipient mice. (C) Absolute number of neutrophils in spleens of recipient mice. (D) Neutrophil frequency in spleens of chimeric mice. (E) Lymph node cells from recipient mice were stimulated ex vivo with PMA and ionomycin and analyzed by intracellular flow cytometry for the production of IL-4, IFN γ , IL-17A, and IL-2.

Notch signaling destabilizes the initiation, but not the maintenance, of Treg differentiation

In addition to causing autoinflammation by promoting the differentiation of multiple inflammatory Th cell subsets, it remained possible that Notch signaling further exacerbated this phenotype by destabilizing Treg cell differentiation. To address this, we assessed the Treg content of CD4-Cre x YFP^{FL/FL} and CD4-Cre x ICN1 mice by flow cytometry. In both the thymus and spleen of CD4-Cre x ICN1 mice, the Foxp3⁺ Treg cell population was decreased, suggesting that CD4-Cre x ICN1 Treg differentiation might be less efficient (Figures 5A-B). To test whether CD4-Cre x ICN1 T cells were refractory to Treg differentiation, CD4⁺ T cells from either CD4-Cre x YFP^{FL/FL} or CD4-Cre x ICN1 mice were activated *in vitro* under Treg polarizing conditions and Foxp3 expression was assessed after five days. While CD4-Cre x YFP^{FL/FL} T cells were capable of inducing Foxp3 expression, CD4-Cre x ICN1 T cells were refractory to Treg differentiation, consistent with *in vivo* findings (Figure 3-6C).

We next sought to establish whether the decreased Treg capacity in CD4-Cre x ICN1 CD4⁺ T cells was cell autonomous, or secondary to the Notch pathway's ability to promote multiple inflammatory Th cell subsets. Splenocytes from SJL, CD4-Cre x ICN1, or SJL/ CD4-Cre x ICN1 mixed bone marrow chimeric mice were analyzed by flow cytometry for Foxp3 expression. Whereas SJL bone marrow recipients exhibited an appreciable Treg population, Foxp3⁺ cells were nearly absent in CD4-Cre x ICN1 chimeras (Figure 3-6D-E). Similarly, the GFP⁺ CD4-Cre x ICN1 compartment in SJL/ CD4-Cre x ICN1 mixed bone marrow chimeras displayed a paucity of Tregs and reduced frequency compared to CD45.1⁺ SJL cells in the same mice, indicating that this

phenotype was likely cell autonomous. These mice also demonstrated a trend toward decreased Tregs within the CD45.1⁺ SJL compartment, albeit still greater than observed amongst GFP⁺ cells, indicating that the Treg deficiency phenotype may be in part due to *trans* effects from the enhanced cytokine production by CD4-Cre x ICN1 T cells (Figure 3-6D-E).

To exclude the possibility that the decreased Treg cell population in the CD4-Cre x ICN1 mice resulted from being exposed to an environment of excess cytokine production, we utilized a Tat-Cre model. This system allows for transient and acute Cre-mediated deletion, thus permitting us to study ICN1^{FL/FL} T cells that develop in an otherwise WT environment *in vivo* and then can be induced to express ICN1 *in vitro*. CD4⁺ T cells from either YFP^{FL/FL} or ICN1^{FL/FL} were treated with Tat-Cre and rested overnight, after which naïve YFP⁺/GFP⁺ T cells were sorted and activated under Treg polarizing conditions. As expected, YFP^{FL/FL} cells were capable of initiating Foxp3 expression under these conditions. In contrast, ICN1^{FL/FL} T cells displayed a decreased Treg differentiation capacity, indicating that the inflammation present in CD4-Cre x ICN1 mice is not the primary cause for the loss in Treg differentiating capacity (Figure 3-6E).

Having confirmed that enforced Notch1 signaling is sufficient to impair Treg differentiation in a cell autonomous manner, we asked whether Notch1 signaling inhibited the initiation of the Treg program or acted to destabilize the maintenance of the Treg identity. To address this, Foxp3-Cre mice were bred to either YFP^{FL/FL} or ICN1^{FL/FL} mice, such that constitutive Notch signaling is induced after the Treg program has been initiated. Naïve CD4⁺ T cells from either Foxp3-Cre x YFP^{FL/FL} or Foxp3-Cre x ICN1^{FL/FL} mice were activated under Treg polarizing conditions and Foxp3 expression was

assessed by intracellular flow cytometry. Unlike the CD4-Cre or Tat-Cre models, in which ICN1 expressing cells were refractory to Treg differentiation, Foxp3-Cre x YFP^{FL/FL} and Foxp3-Cre x NIC^{FL/FL} exhibited a comparable capacity for Treg differentiation in this model, indicating that once Foxp3 expression is initiated, Notch signaling is no longer sufficient to destabilize the Treg program (Figure 3-6F). Thus, Notch signaling acts to impair the initiation of Treg differentiation.

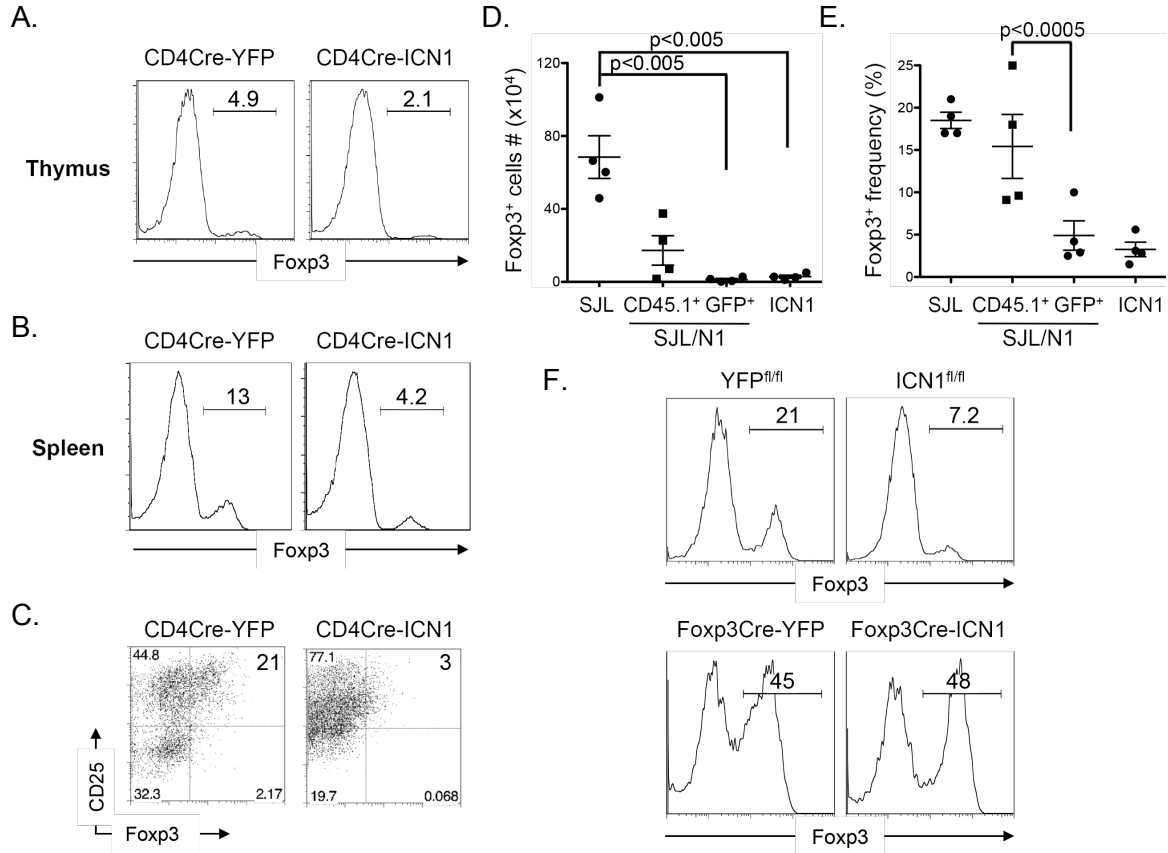


Figure 3-6: Impaired Treg induction in CD4-Cre x ICN^{FL/FL} mice. (A) Frequency of CD4⁺Foxp3⁺ cells in the (A) thymus and (B) spleen of CD4-Cre x YFP^{FL/FL}, CD4-Cre x DNAM1^{FL/FL}, and CD4-Cre x ICN^{FL/FL} mice. (C) CD4⁺ T cells from CD4-Cre or CD4-Cre x ICN^{FL/FL} mice were stimulated as above under Treg polarizing conditions. After 5 days, cells were analyzed by flow cytometry for Foxp3 expression. Bone marrow cells from SJL, CD4-Cre x ICN^{FL/FL}, or both SJL and CD4-Cre x ICN^{FL/FL} mice were adoptively transferred into lethally irradiated SJL recipients. (D) Absolute numbers of splenic Tregs and (E) frequency was assessed by flow cytometry. (F) CD4⁺ cells from YFP^{FL/FL} and ICN^{FL/FL} mice were treated with Tat-Cre, rested for 24 hours, and then YFP⁺ or GFP⁺ CD4⁺ T cells were sorted. T cells were then stimulated as above under Treg polarizing conditions. After 5 days, cells were analyzed by intracellular flow cytometry for Foxp3 expression. (G) CD4⁺ T cells from Foxp3-Cre x YFP^{FL/FL} and Foxp3-Cre x ICN^{FL/FL} mice were stimulated as above under Treg polarizing conditions. After 5 days, Foxp3 expression was analyzed by flow cytometry.

Notch signaling desensitizes activated CD4⁺ T cells to TGF- β and is a key regulator of the Th9-Treg and Th17-Treg axes

We previously demonstrated that Notch signaling acts to potentiate multiple inflammatory Th cell programs by sensitizing activated CD4⁺ T cells to inflammatory differentiating cues. Because enforced Notch1 signaling destabilized Treg differentiation, we hypothesized that the Notch pathway might similarly desensitize activated CD4⁺ T cells to TGF- β , the primary Treg differentiating factor. To test this hypothesis, we activated WT naïve CD4⁺ T cells in the presence or absence of the pharmacological Notch inhibitor GSI (γ -secretase inhibitor), under a series of serially diluted TGF- β concentrations, with IL-2 being held constant. Predictably, as TGF- β was titrated down, decreased Treg differentiation was observed in vehicle control treated (DMSO) cultures (Figure 3-7A). In contrast, Notch-inhibited cultures were resistant to decreased TGF- β concentrations and maintained robust Treg differentiating capacity even in 64-fold diluted TGF- β , where minimal Foxp3 expression is observed in control cultures (Figure 3-7A). As both IL-4 and IFN γ can destabilize Treg differentiation and inhibition of Notch signaling results in decreased production of both these cytokines, the ability of GSI treatment to stabilize Treg differentiation in limiting TGF- β may merely result from decreased IL-4 and IFN γ in these culture. To exclude this possibility, we repeated the TGF- β titration cultures in the presence of neutralizing anti-IL-4 and anti-IFN γ antibodies. While DMSO treated cultures displayed decreased Treg differentiation as TGF- β concentrations became limiting, GSI treated cultures displayed high levels of Treg differentiation at concentrations that failed to support this population in control conditions

(Figure 3-7B). It is worth noting that the effect of GSI at lower TGF- β concentrations was not as robust when neutralizing antibody was present, suggesting that while the dominant effect of Notch inhibition is TGF- β -desensitization, loss of inflammatory cytokine production likely also contributes.

To verify that the enhanced TGF- β sensitivity observed in GSI treated cultures was due to effects on the Notch pathway and not another γ -secretase-dependent process, we repeated the TGF- β titration assay using either Tat-Cre treated YFP^{FL/FL} or DNMAML^{FL/FL} CD4⁺ T cells. Similar to GSI treatment, DNMAML expressing T cells retained an elevated Treg differentiating capacity at limiting doses of TGF- β , whereas YFP^{FL/FL} cells lost Foxp3 expression as TGF- β was diluted in the culture (Figure 3-7C). These data indicate that while Notch signaling sensitizes activated T cells to inflammatory differentiating cues, it concurrently acts to desensitize these cells to TGF- β -derived signals, thus providing a mechanistic basis for the Treg phenotype observed in ICN1 mice.

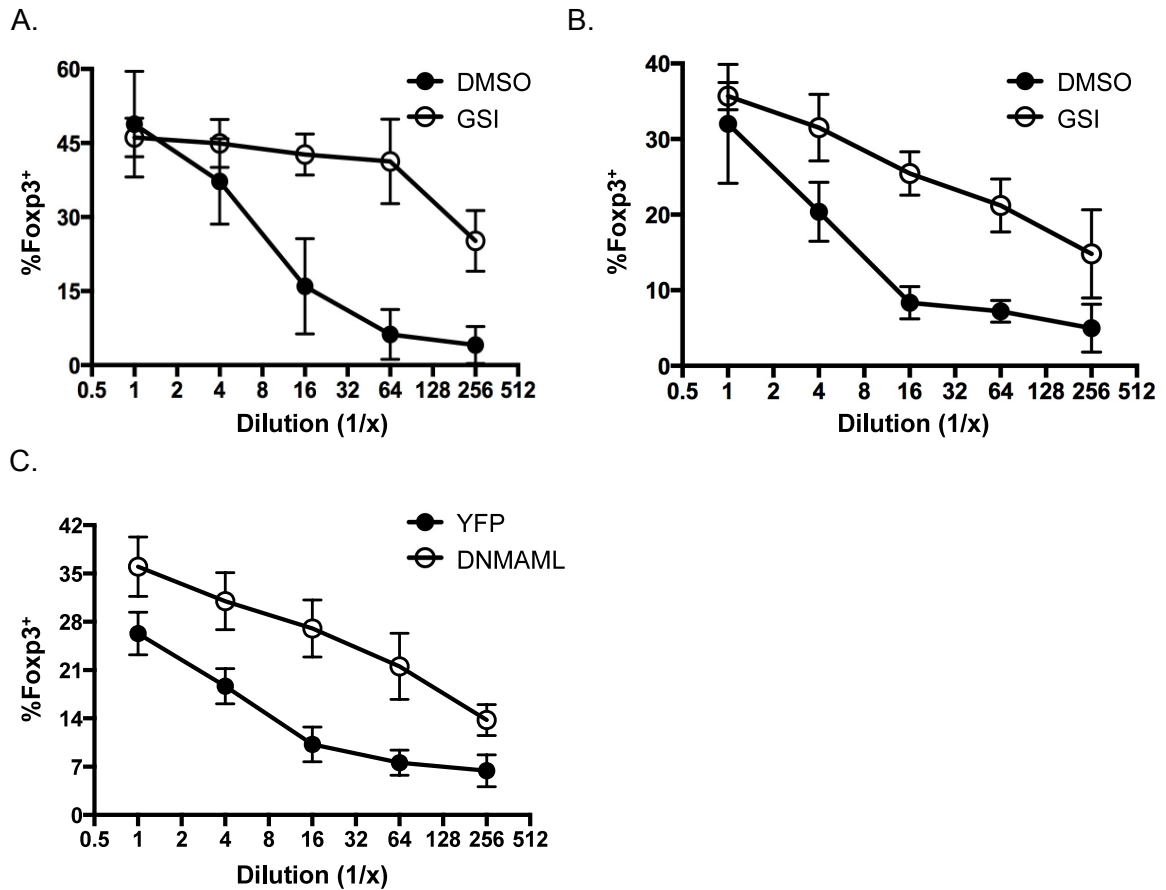


Figure 3-7: Notch signaling desensitizes activated T cells to TGF β . WT CD4⁺ T cells were stimulated as described and then cultured in the presence of either DMSO or GSI with media containing (A) a titration series of four-fold serially diluted TGF β and constant IL-2 or (B) or the same titration series plus anti-IL-4 and anti-IFN γ . After 5 days, cells were analyzed for Foxp3 expression by intracellular flow cytometry. (B) CD4⁺ cells from YFP^{FL/FL} and DNMAHL^{FL/FL} mice were treated with Tat-Cre, rested for 24 hours, and then YFP⁺ or GFP⁺ CD4⁺ T cells were sorted. T cells were then stimulated and then cultured in a titration series of four-fold serially diluted TGF β and constant IL-2. After 5 days, cells were analyzed by intracellular flow cytometry for Foxp3 expression.

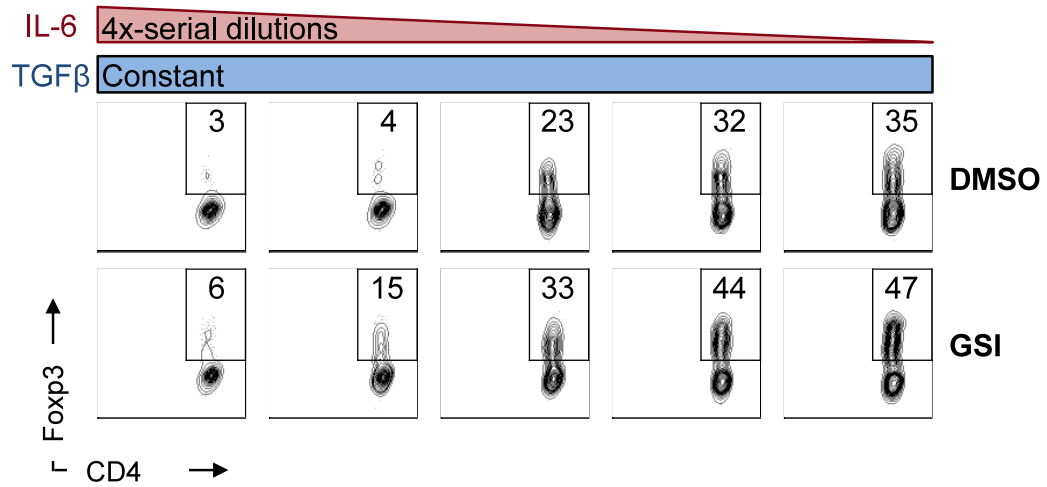
Given the Notch pathway's unique role in regulating T cell sensitivity to both inflammatory and regulatory cues, we predicted that this would place Notch as a key regulator of the Th17-Treg and Th9-Treg axes. The inflammatory Th subsets, Th17 and Th9, each require the presence of both TGF- β and the inflammatory cytokines IL-6 and IL-4, respectively, to initiate their differentiation programs. Thus, as inflammatory cytokines become limiting, Notch signaling should regulate a switch in the choice between an inflammatory or regulatory Th cell fate under these complex cytokine conditions. We previously showed that when IL-6 is serially diluted from Th17 conditions in the presence of GSI that IL-17A production is rapidly lost, compared to vehicle control, however it remained unclear if Notch inhibition can still promote Treg differentiation in conditions that favor inflammatory Th cell differentiation.

To test our hypothesis, naïve CD4⁺ T cells from WT mice were activated under either Th17 or Th9 conditions, with IL-6 and IL-4, respectively, being serially diluted and TGF- β held constant. As expected, titrating IL-6 from the culture in the presence of DMSO resulted in increased Foxp3 expression (Figure 3-8A). When these cells were cultured in the presence of GSI, Foxp3 expression was more readily induced and expressed in a greater fraction of cells at all IL-6 concentrations, suggesting that Notch inhibits Treg differentiation and stabilizes Th17 differentiation in order to lower the cytokine threshold for commitment (Figure 3-8A).

Similar to titrating IL-6, serial dilution of IL-4 resulted in a gradual decrease in Gata3 expression and eventually complete destabilization of Th9 differentiation at 256-fold diluted IL-4 (Figure 3-8B). Unlike Th17 differentiation, which was more readily destabilized at lower concentrations of IL-6, Th9 differentiation remained remarkably

stable when Notch signaling was permitted, with Gata3 being expressed in nearly all cells until IL-4 was markedly diluted. In contrast, when Notch signaling was inhibited, Th9 differentiation was markedly destabilized. Gata3 expression was lost at 4-fold diluted IL-4 in the absence of Notch signaling and the majority of cells were Gata3⁻ at lower concentrations (Figure 3-8B). Conversely, Foxp3 expression was more readily induced at higher concentrations of IL-4 and accumulated as a Foxp3⁺Gata3⁻ population at the lowest concentrations, whereas the majority of Foxp3⁺ cells in control cultures retained Gata3 expression (Figure 3-8B). Altogether these data indicate that Notch signaling plays a critical role in stabilizing inflammatory Th cell differentiation by antagonizing Treg differentiation in the presence of a complex cytokine milieu.

A.



B.

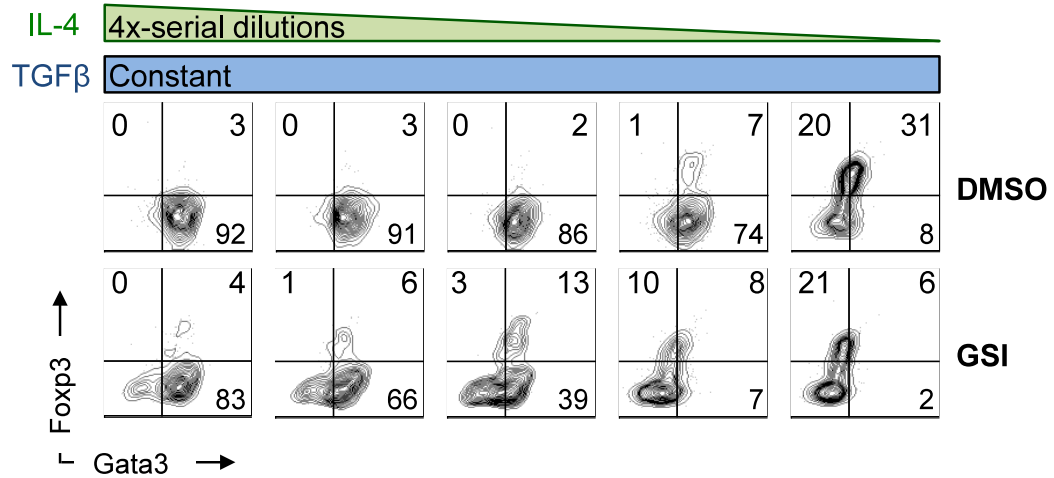


Figure 3-8: Notch is a critical regulator of the Th17-Treg and Th9-Treg axes. WT CD4⁺ T cells were stimulated as described and then cultured in a titration series of four-fold serially diluted (A) IL-6 or (B) IL-4 and constant TGFβ in the presence of either DMSO or GSI. After 5 days, cells were analyzed by intracellular flow cytometry.

Discussion

The Notch pathway frequently functions as a potent instructional pathway, classically involved in bipotential fate decisions during development (Radtke et al., 2010). We recently reported a role for Notch as an unbiased amplifier of multiple inflammatory Th cell subsets, which is mechanistically distinct from its instructional role during T lineage development before β -selection. Here we tested the hypothesis that Notch signaling plays unique roles pre- and post- β -selection by expressing a constitutively active form of Notch1 under the control of CD4-Cre. Unlike studies in which activation of Notch signaling prior to β -selection resulted in T lineage instruction and drove T-ALL onset, CD4-Cre ICN1 mice fail to develop leukemia and instead exhibit a lethal autoinflammatory disorder mediated by a cell autonomous increase in Th1, Th2, and Th17 differentiation. Additionally, our studies revealed a novel role for Notch signaling in inhibiting Treg differentiation, by desensitizing activated CD4⁺ T cells to TGF β .

The role of the Notch pathway as an instructor of normal and malignant T lineage development is well known. Constitutive activation of Notch signaling is sufficient to launch the T lineage program and induce leukemia in multiple models. Early studies showed that retroviral expression of ICN1 in bone marrow progenitors resulted in *de novo* T cell development outside of the thymus, at the expense of B cell development, and ultimately T-ALL onset (Allman et al., 2001; Pear et al., 1996; Pui et al., 1999). Conversely, without the instructive signals derived from Notch, T lineage development fails to occur and B cell development can be observed in the thymus (Han et al., 2002; Maillard et al., 2004; Radtke et al., 1999). Even after progenitors commit to the T

lineage, enforced Notch signaling has the capacity to instruct a T-ALL program. Expression of ICN1 using Lck-Cre, which is active prior to β -selection, results in thymocyte expansion and drives T-ALL, while Lck-Cre x ICN3^{FL/FL} mice similarly exhibit increased thymocyte cellularity and T-ALL (Bellavia et al., 2000; Bellavia et al., 2002; Deftos et al., 2000; Robey et al., 1996).

Our work using CD4-Cre x ICN1^{FL/FL} mice now illustrates that these potent pro-leukemic and instructional activities exerted by the Notch pathway at early stages of T lineage development are lost after β -selection. Cre expression in this model begins after TCR β is expressed and peaks between the DP and SP stages of thymocyte development (Shi and Petrie, 2012; Wolfer et al., 2002). Although all NIC mice analyzed in our study died by 6 months after birth, this was due to a severe autoinflammatory disorder and none of the mice developed leukemia. This finding is consistent with the observation that inactivation of the Notch pathway in the T cell compartment after β -selection results in no gross developmental defects and only yields phenotypes after peripheral T cells are activated (Amsen et al., 2004; Maillard et al., 2004; Tu et al., 2005; Wolfer et al., 2002).

Similar gain-of-function studies have also been used to contend that Notch instructs fates at later stages of T cell development. Retroviral expression of ICN1 in activated T cells was suggested to selectively instruct the differentiation of individual Th cell subsets. Enforced Notch activation in Th cells has been shown to solely instruct Th2 differentiation in multiple studies, while other groups have reported the selective induction of IFN γ and Tbet by intracellular forms of Notch (Amsen et al., 2007; Amsen et al., 2004; Fang et al., 2007; Maekawa et al., 2003; Minter et al., 2005). While our *in vivo*

model of Notch activation agrees insofar that Notch signaling can support both Th1 and Th2 differentiation, we fail to see any selectivity or instruction by Notch signaling with regard to cytokine production by Th cells. Instead, we observe a concurrent and cell-autonomous increase in Th1, Th2, and Th17 differentiation, consistent with our molecular and loss-of-function findings that the Notch pathway simultaneously amplifies the differentiation of multiple inflammatory Th cell subsets(Bailis et al., 2013). It is likely that the discrepancies between our *in vivo* study and previous *in vitro* studies using ICN stems from the fact that Notch signaling acts to sensitize activated T cells to exogenous, inflammatory differentiating cues(Bailis et al., 2013). Thus, the manner in which an *in vitro* differentiation system is established can yield seemingly instructive phenotypes for ICN expression if these conditions have an inherent bias toward Th1 or Th2 differentiation. Indeed, in the same study that ICN1 was shown to instruct Th2 differentiation *in vitro*, loss of Gata3 in this model resulted in Th1 “instruction” by ICN1(Amsen et al., 2007). Finally, these new gain-of-function findings exclude the possibility that our loss-of-function studies masked a dose-dependent instructional role for Notch signaling in Th cell differentiation and show that the Notch pathway is both necessary and sufficient in potentiating multiple fate outcomes in the peripheral T cell compartment, under weak differentiating conditions.

In addition to simultaneously promoting differentiation of multiple inflammatory Th cell subsets, our study also evinces a new role for the Notch pathway in destabilizing Treg differentiation. In keeping with a model in which Notch signaling functions as an integrator of exogenous cues, we find that Notch acts to desensitize activated T cells to environmental TGF β during the initiating of Treg differentiation, but fail to see this effect after Foxp3 is already expressed. Moreover, our mixed bone marrow chimera

experiments indicate this is a cell-autonomous function. Nonetheless, previous studies have argued for a positive regulatory role for Notch in Treg differentiation. In one study, inhibition of Notch signaling using either GSI or an antisense transgene was suggested to impair Treg differentiation (Samon et al., 2008). The differences between our findings may be accounted for by the high levels of ICN observed in the other study's GSI-treated and Notch1-antisense samples. Additionally, our studies used naïve CD4⁺ T cells and antigen presenting cells for our *in vitro* assays, in contrast to the use of total CD4⁺CD25⁻ cells and plate-bound antibody. In another study, Lck-Cre x ICN3^{FL/FL} mice displayed a substantial expansion of CD25⁺Foxp3⁻ cells, but also exhibited a roughly three-fold expansion of CD25⁺Foxp3⁺ cells with enhanced suppressor function. This expanded Treg population is lost when ICN3-transgenic mice are crossed to *Ptcra*^{-/-} mice, indicating that these cells are likely naturally occurring Tregs and may suggest distinct roles for Notch in thymic and peripheral Treg populations (Anastasi et al., 2003; Campese et al., 2009). It is also worth noting that autoinflammatory disorders have not been observed in T cell-specific conditional loss-of-function Notch models, as would be predicted if Treg differentiation were impaired, and instead defects in inducing inflammation are present (Radtke et al., 2013). Our studies are consistent with a role for Notch as a negative regulator of Treg differentiation as autoinflammation results from Notch1 gain-of-function.

In summary, our gain- and loss-of-function data evince a role for Notch as an unbiased integrator of exogenous differentiation cues that act to sensitize T cells to inflammatory signals and desensitize T cells to regulatory signals. This model is consistent with our observation that the Notch pathway is a key regulator of the balance of regulatory and inflammatory Th cell populations that are activated under complex cytokine mixtures,

such as Th9 and Th17 differentiation. While a role for Notch in Th9 and Th17 differentiation has been previously reported (REFS), we find that loss of Notch signaling not only destabilizes the differentiation of these subsets, but also enhances Treg differentiation under conditions that otherwise would not favor this population (Elyaman et al., 2012; Keerthivasan et al., 2011; Mukherjee et al., 2009). Indeed, Notch inhibition concurrently results in impaired Th17 differentiation and enhanced Treg differentiation in experimental autoimmune encephalomyelitis (Bassil et al., 2011; Keerthivasan et al., 2011; Sandy et al., 2013). Moreover, inhibiting the Notch pathway has also been found to simultaneously impair Th1, Th2, and Th17 differentiation while enhancing Treg differentiation in a mouse model of graft-versus-host disease (Tran et al., 2013; Zhang et al., 2011). Our findings now provide a mechanistic basis for these observations and further highlight the utility of Notch inhibition as a therapeutic for inflammatory disorders.

CHAPTER 4: Discussion and Future Directions

Lineage development is directed both by extrinsic environmental cues, in the form of soluble mediators and cell-surface molecules, and intrinsic regulation of transcription at the level of transcription factors, chromatin conformation, and epigenetic modification. The context and manner in which a developing cell integrates this wide range of inputs ultimately dictates the fate choices that the cell will make. Within the hematopoietic system, T lineage development is one of the best characterized developmental pathways, with both extrinsic and intrinsic mediators known for multiple well defined developmental stages (Rothenberg et al., 2010). Yet despite our wealth of knowledge on the development of this essential immune population, we have little understanding of how many of the intrinsic mediators of T lineage development exert distinct functions at multiple stages in the T lineage progression. For example, Gata3 is understood to be essential for early T lineage commitment of all $\alpha\beta$ -T cells, the induction of CD4⁺ SP (but not CD8⁺ SP) thymocyte development, and finally the selective instruction of Th2 differentiation at the expense of other Th cell fates (Rothenberg and Scripture-Adams, 2008). Moreover, Gata3 has been found to regulate distinct sets of transcriptional targets at each of these stages, an observation that cannot solely be explained by transcription factor concentration and/or motif binding affinity (Wei et al., 2011). Here I present findings that illustrate how the Notch pathway similarly exerts unique functions at early and late T cell development. While Notch signaling displays strong instructional capacity prior to the β -selection checkpoint, I find that Notch signaling lacks this ability thereafter and instead acts to potentiate multiple Th cell fates by sensitizing activated CD4⁺ T cells to exogenous differentiating cues, thus functioning as a signal integrator. The

implications for these findings on Notch signaling in T lineage development, T-ALL transformation, and development of non-T populations within the hematopoietic system are discussed in this chapter.

Two paradigms of Notch signaling, one lineage

The Notch pathway is a potent lineage instructor during early hematopoietic development. Enforced Notch signaling in bone marrow progenitors is sufficient to divert cells with lymphoid potential away from the B lineage and launch the T lineage program, while loss of Notch signaling renders the thymus barren of its ability to foster T lineage development (Allman et al., 2001; Pear et al., 1996; Pui et al., 1999; Radtke et al., 1999). Even in the absence of an ICN transgene, Notch ligand stimulation is sufficient to divert myeloid progenitor cells into the T lineage (Chi et al., 2011). This instructive capacity of Notch is so pronounced that T lineage cells develop outside of the thymus when signaling is constitutively on, illustrating that Notch signaling itself, not other components of the thymic microenvironment, dictates entry into T development.

Mechanistically, Notch signaling is understood to instruct T cell development by coordinating the expression of genes that directly program the T lineage identity as well as genes that function to restrict alternate fates. The TCR is fundamental to the T cell identity and core function and the Notch pathway is understood to regulate several key stages of its assembly. As discussed in Chapter 1, Notch signaling is essential for the first stages of TCR assembly. Notch both directly and indirectly regulates *Ptcra*, by binding to the *Ptcra* promoter to drive transcription and by promoting the splicing of Ikaros isoforms that lack DNA binding capacity (Bellavia et al., 2007; Reizis and Leder,

2002). Moreover, RBPJ-deficient thymocytes display impaired TCR β rearrangement, suggesting that Notch regulates pre-TCR signaling at the level of both pTa and TCR β (Ciofani et al., 2006; Wolfer et al., 2002). In addition to regulating TCR assembly, Notch signaling controls the expression of three of the most important T lineage commitment factors: *Gata3*, *Tcf7* (Tcf1), and *Bcl11b* (Rothenberg, 2012; Rothenberg et al., 2010). All three of these factors are required for early T development and, in addition to regulating a network of key T lineage identity genes, Tcf1 and Bcl11b act to restrict alternate lineage programs. While Bcl11b limits NK and myeloid potential and terminates gene expression associated with the stem/progenitor program, Tcf1 plays an important role in restraining B cell development (Ikawa et al., 2010; Li et al., 2010a; Li et al., 2010b; Weber et al., 2011). Furthermore, the canonical Notch target Hes1 has recently been shown to constrain myeloid development within the thymus (De Obaldia et al., 2013). Finally, Notch further promotes T lineage development by providing a growth and survival advantage for T committed cells, *via* the direct regulation of two essential thymocyte survival factors: *Il7ra* and *Myc* (Gonzalez-Garcia et al., 2009; Wang et al., 2014; Weng et al., 2006). Thus, the Notch pathway instructs T lineage development by promoting the assembly of the TCR, launching the core T lineage transcriptional network, restricting alternate fates, and promoting the outgrowth of committed cells.

In contrast to the multilayered instructional capacity of Notch during early T development, the work presented in Chapters 2 illustrates the lack of instruction by the same pathway during late-stage T development. Like its regulation of T lineage defining factors such as the TCR and the core T lineage transcriptional network, Notch signaling similarly regulates the signature effector proteins and transcription factors of Th cells.

However, instead of supporting a single Th cell subset, Notch simultaneously binds and directly regulates *Il4*, *Ifng*, *Il17a*, *Gata3-1a*, *Tbx21*, and *Rorc*, early after T cell activation. This activity appears to only be essential when environmental differentiation cues are limiting, as Th cells can differentiate successfully in the absence of Notch at high cytokine concentrations, whereas the requirement for Notch signaling in T lineage entry is absolute. The gain-of-function studies presented in Chapter 3 further illustrate the Notch pathway's ability to promote the differentiation of multiple Th cell populations *in vivo*, in contrast to the selective induction of T cell development when Notch signaling is enforced prior to β -selection (Allman et al., 2001; Pear et al., 1996; Pui et al., 1999). Insofar as the Notch pathway restricts alternate fates during Th cell differentiation, Treg induction is destabilized in the face of constitutive Notch signaling, however residual Treg differentiation capacity persists. This is in contrast to the complete loss of B cell potential in ICN1 transduced bone marrow cells, suggesting that, while the Notch pathway does retain properties of lineage restriction in peripheral T cells, this activity is modest compared to its function in thymocyte development (Pui et al., 1999).

Finally, the extent to which Notch signaling provides a survival or proliferative advantage to signal receiving cells during Th cell differentiation remains less clear. Dll4 stimulation can enhance T cell survival following activation and mice deficient in components of the Notch pathway display decreased proliferation and survival following immunization with adjuvant and peptide (Helbig et al., 2012). Additionally, another report described an important role for Notch signaling in promoting T cell proliferation *via* Myc, downstream of the TCR (Guy et al., 2013). While these two studies suggest that Notch can play a role in peripheral T cell growth and survival, other loss-of-function Notch studies in peripheral T cells have reported no effect of Notch on T cell proliferation following

activation, suggesting that Notch effects on these processes are likely to be context dependent, much like the relationship between Notch and cytokines (Amsen et al., 2004; Bailis et al., 2013; Minter et al., 2005; Tu et al., 2005). Nonetheless, both these studies failed to report any selective preference of Notch signaling in promoting the survival of individual Th cell subsets, suggesting that while the pathway may similarly regulate growth and survival during early and late T lineage development, this activity does not impart any instructive capacity to Notch during Th cell differentiation as it does during T lineage entry.

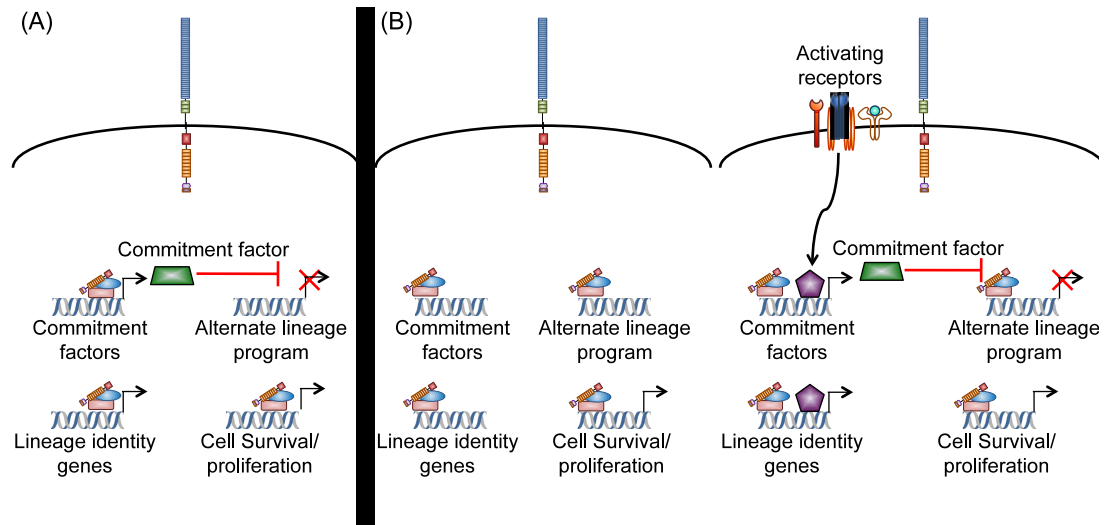


Figure 4-1: Two paradigms of Notch signaling. (A) Notch can function as a lineage instructor during development, as seen in early thymocyte development. The Notch transactivation complex is capable of binding and directly activating genes that define lineage identity (i.e. *Tcrb*), genes that govern lineage commitment (i.e. *Bcl11b*), and genes that control cell survival and proliferation (i.e. *Myc*). In turn, lineage commitment factors suppress alternate fate potentials and survival factors promote the outgrowth of committed cells, enforcing lineage identity. (B) Notch also has the capacity to function as an integrator of exogenous differentiating cues, as seen in Th cell differentiation. In the absence of other activating signals, the Notch transactivation complex binds to lineage commitment and identity genes for multiple lineage programs (i.e. *Gata3*, *Tbx21*, and *Rorc*) in an impartial manner, sensitizing them to transactivation by other pathways. Upon receiving lineage-biasing signals, transcriptional activators are recruited to genes that promote the differentiation of a single lineage (i.e. *Tbx21* only) by synergizing with Notch complexes at those sites. Subsequently, the activated lineage commitment factors repress transcription at loci for alternate lineages, reinforcing differentiation. During this process, Notch also provides cell survival and growth signals to all differentiated cells, regardless of lineage. In this setting, Notch remains capable of synergizing with new activating signals that may re-differentiate this cell to an alternate fate.

Rather than supporting an instructional model, the data presented here suggest the Notch pathway acts to integrate a wide array of environmental cues into a coherent transcriptional program. I have shown this at the level of cytokine sensitization and the ability of Notch to synergize with components of these pathways at Th cell signature gene loci, however data from other groups indicate that Notch can similarly integrate signals from other pathways. As mentioned above, Notch signaling can act directly downstream of the TCR. Signal transduction downstream of the TCR is mediated *via* the phosphorylation of multiple conserved motifs (ITAMs) found on CD3 proteins (Love and Hayes, 2010). Upon T cell activation, Guy and colleagues detected the recruitment of Notch1 and components of the Notch cleavage machinery to the TCR, in a manner dependent on high ITAM multiplicity. Given Notch signaling was also found to induce *Myc* transcription and *Myc* expression was found to be reduced in cells with low ITAM multiplicity or activated with weak TCR agonists, it was postulated that Notch signaling directly couples proliferation and cell growth to TCR signal strength, and thus functions as a rheostat for activation as dictated by antigen affinity (Guy et al., 2013). In addition to acting directly downstream of the TCR, Notch signaling has also been suggested to interact with other critical T cell activation pathways. Notch inhibition after T cell activation impairs sustained activation of NF- κ B and ICN1 may even directly interact with components of NF- κ B signaling. Moreover, Notch was found to cooperate with NF- κ B and regulate transcription at the *Ifng* promoter, illustrating its ability to integrate signals from other pathways to mediate transcription of key T cell effector genes, much like we observed for Notch and Tbet (Shin et al., 2006). Similarly, Smad3 directly interacts with Notch1 and the two factors have been shown to cooperatively promote *Il9* transcription (Elyaman et al., 2012). Notch inhibition is also associated with a hyporesponsive phenotype in CD4⁺ and CD8⁺ T cells from a mouse model of GvHD. These cells display

decreased NF- κ B and Ras/MAPK signaling and increased expression of negative regulators of T cell activation, such as *Dgka*, *Cblb*, and *Pdcd1*, suggesting that Notch signaling may act as a global integrator of activating signals (Sandy et al., 2013). While Notch signaling has been found to interact with these pathways and others (such as PI3K, Hedgehog, Wnt, and mTOR) in cancer and other cell types, the extent to which Notch collaborates with the downstream transcriptional effectors from these networks in activated T cells has not been evaluated and is an area of investigation for future studies (Aster et al., 2011; Takebe et al., 2011).

Although this thesis reports findings indicative of a non-instructional role for Notch during Th cell differentiation, this observation has only been made at the population level. Our ChIP and GSI-washout data demonstrate that Notch can bind and directly regulate the signature cytokine and transcription factor genes for Th1, Th2, and Th17 cells, but they do not prove that this occurs simultaneously in the same cell. It remains possible that at the single cell level, the Notch complex may display Th cell type specific activity, such that Notch only binds to *Ifng* and *Tbx21* in cells initiating Th1 differentiation, but not in cells that have begun Th2 or Th17 differentiation. While this would still entail the Notch pathway's involvement in the differentiation of multiple populations from the same progenitor, in contrast to thymocytes, it would be more consistent with what has been observed for Notch in earlier stages of development: Notch selectively regulates cell-type defining genes in individual cells, these targets (e.g. Tbet) coordinate core transcriptional networks and restrict alternate fates, and Notch then selects for differentiated cells by promoting growth and survival. Single-cell array or RNA-seq studies performed on GSI-washout samples will go a long way to addressing this,

however until ChIP technology becomes reliable in resolving transcription factor binding at the single-cell level, these questions will likely remain difficult to directly address.

Implications for Notch signaling and T-ALL

The gain-of-function studies performed in Chapter 3 not only highlight a switch in Notch instructional activity during normal development, before and after β -selection, but also demonstrate a striking and concurrent switch in Notch oncogenic activity in promoting malignant development. Multiple studies have shown that activation of Notch signaling before β -selection, either using retroviral transduction or Lck-cre, results in highly penetrant, rapid onset, and lethal T-ALL (Allman et al., 2001; Bellavia et al., 2000; Bellavia et al., 2002; Deftos et al., 2000; Pear et al., 1996; Pui et al., 1999; Robey et al., 1996). The data in Chapter 3 show that activation of Notch after β -selection, using CD4-cre, fails to result in T-ALL and instead promotes the differentiation of multiple inflammatory Th cell subsets, while destabilizing Treg development (Figure 4-2). These findings raise fundamental questions about the mechanisms Notch signaling utilizes to induce T-ALL.

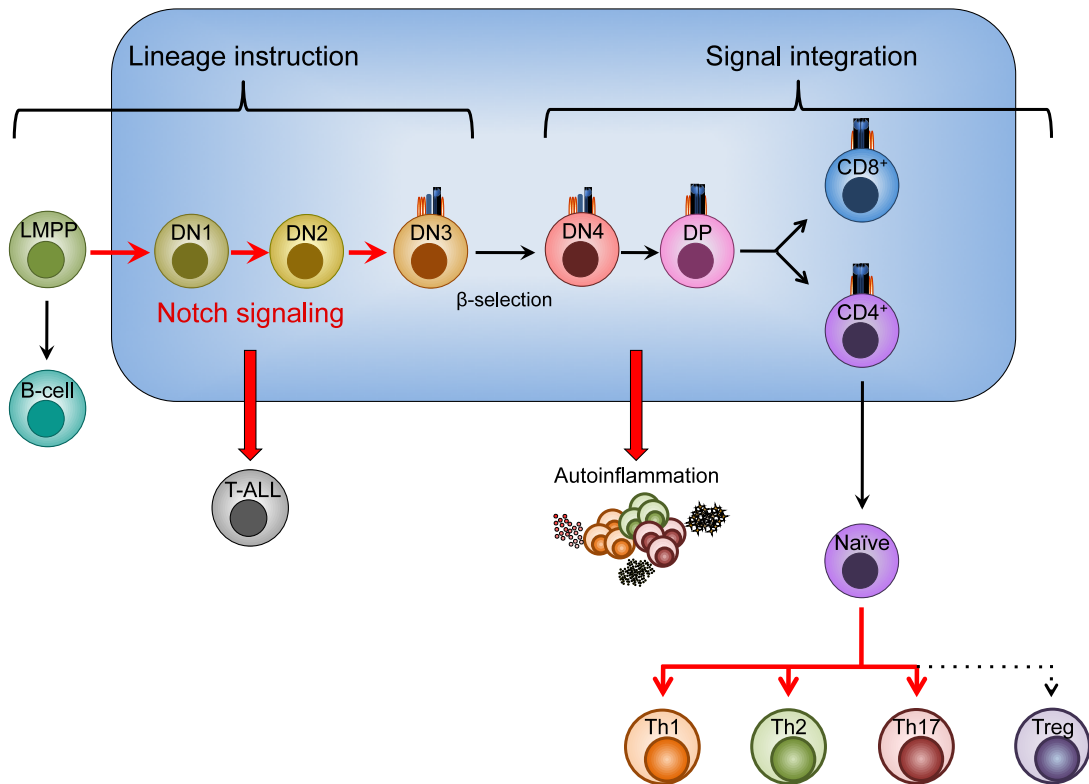


Figure 4-2: Notch signaling mediates distinct functions pre- and post- β -selection. Prior to β -selection, Notch signaling is a robust instructor of cell fate. Activated Notch signaling instructs T lineage development at the expense of B cell development, and hyper-activation of the pathway diverts progenitors into a malignant developmental pathway. After β -selection is completed, Notch signaling is turned off for the duration of thymic development. If Notch signaling is enforced during this period, it is incapable of launching a T-ALL program. Instead, maturing T cells readily activate and differentiate into multiple inflammatory Th cell subsets that in turn promote autoinflammation. Similarly, when Notch signaling is naturally initiated after a naïve CD4⁺ T cell becomes activated, the pathway acts to integrate environmental differentiation cues in a manner that sensitizes activated cells to inflammatory Th cell differentiation and destabilizes Treg differentiation.

Notch is thought to promote T-ALL onset through its ability to regulate multiple pro-survival and pro-proliferative pathways. Among these genes, c-Myc has been identified as the critical Notch target in T-ALL, both necessary and sufficient for Notch driven leukemia (Palomero et al., 2006; Sharma et al., 2006; Weng et al., 2006). Additionally, Notch signaling has been shown to regulate the NF- κ B and AKT pathways to promote malignancy, though neither is sufficient for T-ALL (Landor et al., 2011; Lee et al., 2012; Palomero et al., 2007; Vilimas et al., 2007). One possible explanation for the inability of hyper-active Notch signaling to induce leukemia after β -selection is that one or more of these critical T-ALL targets are inaccessible to Notch at later stages of development. In particular, we have identified a long-distance, Notch dependent *Myc* enhancer that is active in both in DN3 thymocytes and T-ALL and required for Notch activation of *Myc* (in submission). It will be interesting to test whether Notch can still occupy this element, whether the enhancer continues to loop to the *Myc* promoter, and/or whether this enhancer remains active after β -selection. In addition to local studies at the *Myc* enhancer, ChIP-seq analyses for Notch1 and RBPJ comparing ICN1 transduced bone marrow progenitors and DN3 cells from Lck-cre x ICN1^{FL/FL} mice to DP and peripheral CD4⁺ and CD8⁺ cells from CD4-cre x ICN1^{FL/FL} mice will go a long way to clarifying Notch target accessibility before and after β -selection. Combining this approach with RNA-seq and ChIP-seq for various histone modifications in the above populations versus control cells (i.e. Lck-cre x ICN1^{FL/FL} DN3 cells versus Lck-cre x YFP^{FL/FL} DN3 cells) will help resolve the network of genes that the Notch pathway is capable of reprogramming at multiple stages of development, providing functional relevance to Notch1 and RBPJ ChIP-seq findings.

Outside of target accessibility, another potential explanation for the dichotomy observed in Notch signaling's oncogenic potential during T lineage development is that obligate co-factors are only present prior to β -selection. Between the DN2 and DN3 stages, the transcription of many stem/progenitor legacy genes is extinguished, a large portion of which have known oncogenic roles (Rothenberg et al., 2010). Of particular importance, *TAL1* mutations occur in approximately 60% of T-ALL cases and its expression is markedly downregulated in early thymocyte populations, along with its binding partners *LMO1* and *LMO2*. Together, TAL1 and LMO proteins are understood to synergistically cooperate to promote T-ALL, if their expression is sustained (Chervinsky et al., 1999; Larson et al., 1996; Ono et al., 1997; Rothenberg et al., 2010; Van Vlierberghe and Ferrando, 2012). Notch1 mutations spontaneously occur in TAL1/LMO driven T-ALL mouse models, suggesting that the TAL1/LMO pre-leukemic thymocyte population is a highly permissive setting for oncogenic Notch (O'Neil et al., 2006). A recent and elegant study using cell competition to drive the first and only spontaneously occurring T-ALL model in WT thymocytes not only recapitulates the mutations and oncogenic progression found in human T-ALL, but also clearly illustrates that Notch mutations occur secondary to sustained TAL1/LMO expression (Martins et al., 2014). Together these findings suggest that Notch signaling may only be able to transform pre-leukemic populations, which is most commonly established by TAL1/LMO, and the factors needed to create this pre-leukemic state may only be expressed prior to β -selection.

CRISPR technology now enables us to rapidly model and study complex transgenic combinations and could be utilized to test this hypothesis. Generation of Lck-cre x *Tal1*^{FL/FL}*ICN1*^{FL/FL} mice, such that *Tal1* is deleted in DN2 cells and *ICN1* is expressed, should reveal whether or not the TAL1 complex is necessary for transformation by

Notch. In a converse experiment, CD4-cre x tgTal1^{FL/FL}tgLmo1^{FL/FL}ICN1^{FL/FL} mice could also be generated, such that TAL1, LMO1, and ICN1 are constitutively expressed after β -selection, and would test the hypothesis that ICN1 fails to transform T lineage cells later in development due to the lack of stem/progenitor legacy factors needed to establish a pre-leukemic population. Although the mechanism responsible for the switch in the Notch pathway's oncogenic potential during T cell development remains unclear, the findings presented in this thesis provide the framework needed to address these questions in the future.

Revisiting the linear instruction model of Th cell differentiation

As discussed in Chapter 1, research over the past decade has increasingly challenged Mossman and Coffman's Th1/Th2 paradigm and the linear instruction model. The identification of non-classical subsets such as Th17 and Treg cells, as well as the putative Th9 and Tfh populations, and the observation that Th cells can interconvert has not only invalidated a simplified bipotential model, but also directly questions whether Th cell subsets represent true lineages defined by rigid programs. While different cytokines clearly play a role in patterning and stabilizing individual Th cell programs, research has often overlooked the existence of a core network of activating signals shared by all Th subsets, derived from the TCR, costimulation, and as presented in this thesis, Notch receptors, as the linear instruction model discounts viewing Th cells as a single population. Researchers have attempted to apply the linear instruction model to all three of these pathways, however TCR, costimulation, and now Notch signaling have all been shown to influence the differentiation of multiple subsets in highly context dependent manner, rather than displaying any instructive capacity. From this perspective, it may be

more appropriate to view Th cells as a single lineage with the capacity to flexibly adopt multiple functional modules that form semi-stable effector states (Figure 4-3).

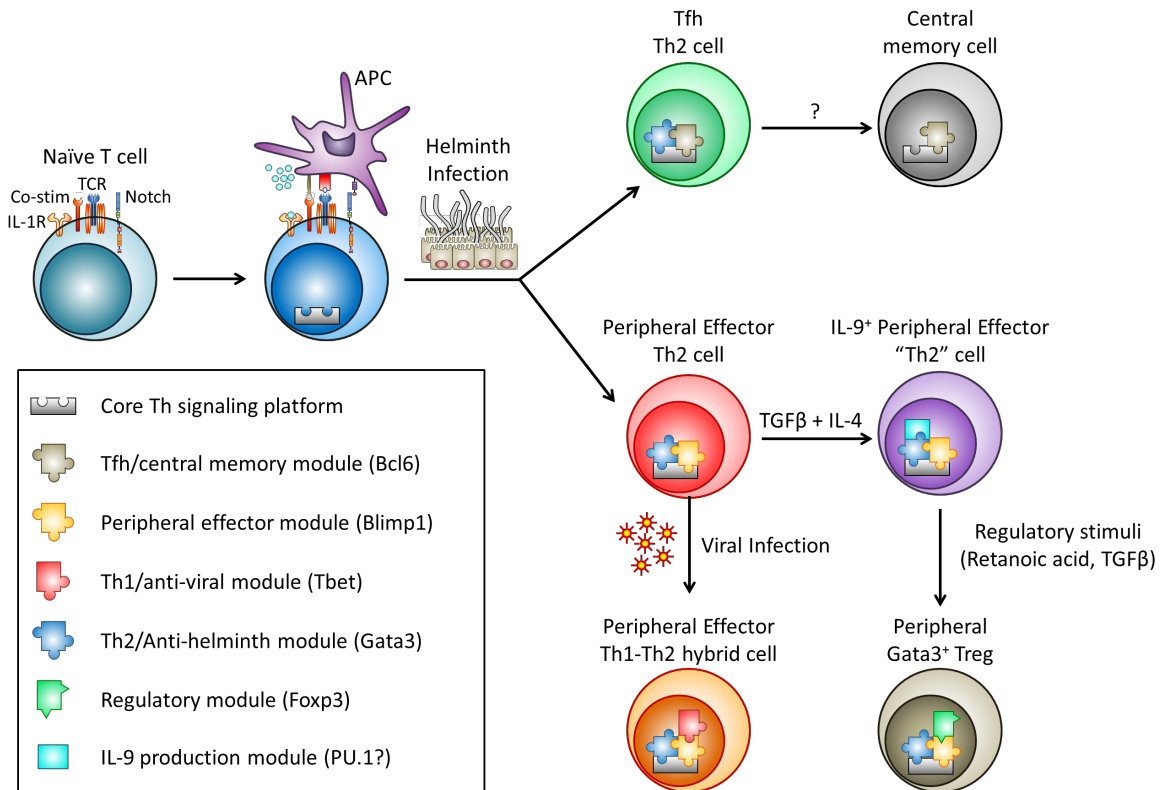


Figure 4-3: Hypothetical “modular” model of Th cell differentiation. When a naïve T cell sees its cognate antigen on an MHC-II-bearing antigen presenting cell (APC), it receives signals through a core Th cell signaling network consisting of the IL-1R, co-stimulatory receptors, the TCR, and Notch receptors. Together, the terminal transcriptional effectors of these pathways construct a core platform of epigenetic architectures and transcription factor complexes, such that the cell is receptive to integrate unique signals from the given inflammatory environment. Shown is a hypothetical Th cell response during helminth infection. Signals derived from invading parasites are integrated into the core Th signaling platform and activate an anti-helminth Th cell response (classically Th2). Gata3 and other transcriptional effectors activate a modular network of transcriptional and epigenetic modifications that stabilize a Th2-like phenotype. Depending on other signals received by these cells, they may either activate a program that homes them to secondary lymphoid organs *via* a Bcl6-orchestrated network, or traffic to the site of inflammation using a Blimp1-dependent effector module. During the course of infection, these peripheral effector Th2-like cells may be exposed to factors that license the production of auxiliary cytokines, such as IL-9, and these cells may further be stimulated to turn on a Foxp3-regulated regulatory T cell transcriptional module to further tailor their function. If a secondary infection occurs, these peripheral Th2-like effector cells may adopt additional pathogen-specific programs, such as an anti-viral Th1 module, that enables these cells to participate in complex immune responses. Finally, some of the cells operating both a Th2-like and a Tfh module will turn off their anti-helminth response, while maintaining the Bcl6-governed module, and become central memory cells.

Recent studies looking at the epigenetics of lineage differentiation highlight how Th cell subsets are better represented as a single lineage with a large common regulatory network rather than multiple distinct populations. A seminal epigenetic study looking at global histone modifications in the various Th cell subsets found that few, if any, unique epigenetic architectures existed amongst the individual Th cell subsets. Only 3-7% of H3K4me3 labeled genes, denoting primed or active promoters, were unique to Th1, Th2, Th17, or Treg cells, with these subtypes diverging by 5-10% in terms of H3K27me3 labeling, denoting primed or silenced genes. By comparison, thymus-derived natural Tregs diverged by 28% and 22% for H3K4me3 and H3K27me3 from the other Th cell populations, suggesting that differentiated Th cells are more similar than different, even when compared to a closely related population. Moreover, the signature genes for alternate Th cell programs were held in a poised chromatin conformation, indicating that these loci could readily be expressed upon stimulation. This is distinct from the loss of gene poising of lineage-specific factors during the differentiation of *bona fide* hematopoietic lineages (Cedar and Bergman, 2011; Wei et al., 2009). Outside of the canonical signature genes (i.e. *Ifng* and *Tbx21* for Th1, *Il4* and *Gata3* for Th2, etc), global gene profiling revealed a high degree of similarity in Th cell transcription, even with regard to the transcription of genes thought to be selectively associated with only one Th cell subset (e.g. appreciable *Il5* transcripts were observed in Th1, Th2, and Th17 cells) (Wei et al., 2009).

A more recent study analyzed enhancer usage amongst the different Th cells subsets and once again found a remarkable amount of epigenetic homology amongst these populations. Using p300 binding as a surrogate for enhancer usage, Vahedi and colleagues found that unlike distinct lineages such as macrophages or embryonic stem

(ES) cells, which respectively only shared ~10% and <1% of their enhancer usage with differentiated Th cells, Th1 and Th2 cells shared 65% and 55% of their enhancer usage together, despite these two cell types being considered mutually exclusive states with distinct transcriptional profiles. Not only do these cells exhibit similarity in global enhancer usage, but they also share a common transcription factor regulatory network. Even when enhancers specific to either Th1 or Th2 cells were analyzed for transcription factor motif enrichment, the top three motifs were shared by both subsets: AP1, RUNX, and NFAT. This particular finding further highlights the usage of a common signaling network by all Th cells, as AP-1 and NFAT are downstream effectors of TCR and co-stimulation, respectively. In addition to these core-signaling factors, the GAS-3 motif, which is bound by STAT1 and STAT4, was found to be one of the most enriched motifs at both Th1- and Th2-specific enhancers (Vahedi et al., 2012). While STAT1 and STAT4 are downstream of the Th1-associated cytokines IFN γ and IL-12, type-1 interferons can also activate STAT1 and it is interesting to speculate that these data implicate this important family of inflammatory mediators as a component of the core Th signaling network. In contrast to the shared motifs used by Th1 and Th2 cells, even at subset-specific loci, a similar motif enrichment analysis performed at the enhancers of B cells, erythrocytes, CD4⁺ T cells, ES cells, and liver cells found almost no homology in transcription factor motif enrichment (Heinz et al., 2010). Collectively, epigenetic and transcriptional profiling of the Th cell subsets shows that, while these populations indeed represent unique functional programs, they display a striking degree of homology that is utterly lacking when similar analyses are performed on *bona fide*, distinct lineages.

Viewing Th cells as a single lineage with a broad shared signaling network not only is more compatible with epigenetic and transcriptional data, but is also more in line with our

current understanding of Th cell plasticity. In this context, the “master regulators” can be understood to impart bundled and stackable effector properties. Tbet, Gata3, and Ror γ t may be better appreciated as pathogen-specific programs tailored to home effector cells to the relevant tissue where they can properly eliminate invaders, rather than lineage reprogramming factors. These pathogen-specific programs could be overlaid with a regulatory program (Foxp3), a germinal center/central-memory program (Bcl6), a peripheral tissue effector program (Blimp1), or licensed to produce additional accessory cytokines (IL-9), in order to tailor nuanced local responses. Given unique classes of pathogens require different immune responses and it is necessary to maintain a stable, coherent response until a pathogen is cleared, it is not surprising that the pathogen-programming factors (Tbet, Gata3, and Ror γ t) tend to be expressed independently of one another and we fail to observe the spontaneous introversion of Th cells in classic infection models. Moreover, as Th cells have such a robust core signaling network, there is a need for factors that permit T cells to efficiently adopt individual anti-pathogen programs. In this light, the findings reported in Chapters 2 and 3 demonstrate that the Notch pathway is a component of the core Th cell signaling network that allows for the rapid diversion of an activated cell into one of these semi-stable, pathogen-specific functional programs (Figure 4-4). Thus Notch is not essential for differentiation if exogenous factors can robustly activate these programs alone, but inhibition of Notch signaling during immune responses with weak or complex inflammatory milieus results in the marked destabilization of Th cell differentiation, as seen in a wide variety of models (Billiard et al., 2012; Fang et al., 2007; Jiao et al., 2014; Park et al., 2013; Tran et al., 2013; Zhang et al., 2011).

While the classical linear instruction model remains useful for conceptualizing the unique properties of each Th cell subtype, it is inadequate for predicting the behavior of the signaling molecules and transcription factors participating in Th cell differentiation, other than those involved in cytokine signaling. By modeling Th cell differentiation as a single lineage capable of adopting multiple, overlaid functional modules, there becomes less of a need to understand how these programs are rigidly enforced and a greater need to understand how the multitude of signals that activated T cells receive are coherently integrated to give the illusion of distinct lineages. Moreover, increased emphasis is needed to understand how exogenous factors modify the outputs derived from the core signaling network, rather than looking for how these factors lead to a linear in-put/out-put within a single signaling pathway.

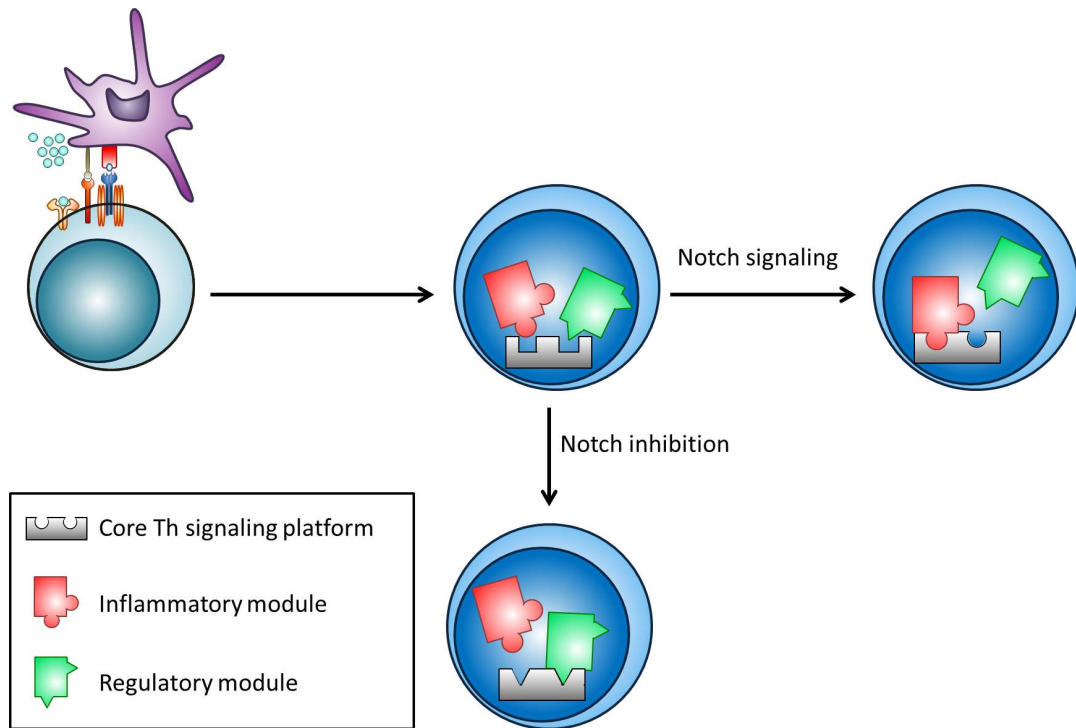


Figure 4-4: Notch and the hypothetical “modular” model. Notch acts to promote the integration of inflammatory modules into the core Th signaling platform, while destabilizing the integration of regulatory modules. When CD4⁺ T cells are activated, the core Th signaling platform inefficiently integrates both inflammatory and regulatory modules. When Notch signaling is engaged, inflammatory modules are readily integrated, while regulatory modules are excluded. In contrast, when Notch signaling is inhibited, inflammatory modules are poorly integrated and regulatory modules are utilized.

Applying the signal integration model of Notch to other developmental processes

It is not surprising that the first Notch-dependent phenotypes described were those involved in fate choice decisions, as these phenotypes provided robust all or none results in genetic studies (Guruharsha et al., 2012). However, recent work on Notch signaling has highlighted how this classically instructive pathway can be involved in the development of multiple closely related lineages in the hematopoietic system. Innate lymphocytes (ILCs) are an innate immune population that shares both functional and developmental properties with T lineage cells. Not only are ILCs subfractionated using identical functional criteria as Th cells (with ILC1s producing Th1-like cytokines, ILC2s producing Th2-like cytokines, and ILC3s producing Th17-like cytokines), but they also share some of the core T lineage transcriptional network. Gata3, Tcf1, and Notch signaling have all been shown to play roles in ILC development and it has been suggested that this implies an evolutionarily conserved transcriptional network shared between ILCs and thymocytes (Tait Wojno and Artis, 2012; Yang et al., 2013). Notch signaling is thought to be involved in both ILC2 and ILC3 development. Two different populations of ILC3s, lymphoid tissue inducer cells (LTi) and IL-22 producing ILCs (ILC22s), require Notch signaling during development and ILC2 development is similarly dependent on Notch signaling both *in vitro* and *in vivo* (Cherrier et al., 2012; Lee et al., 2011; Wong et al., 2012b; Yang et al., 2013). If one envisions that the role Notch plays in ILC development occurs along similar lines as its unbiased role in Th cell differentiation, then one would predict that Notch signaling does not instruct individual ILC subsets, but rather allows for efficient differentiation and/or function of all three ILC subtypes when conditions are otherwise not permissive. Notably, ILC22 development is only impaired in

the lamina propria, but not the Peyer's patches, in the absence of RBPJ, consistent with the notion that the requirement for Notch may depend on whether the exogenous differentiating cues present in a given environment are limiting, rather than an absolute necessitation for the pathway (Lee et al., 2011). While it remains to be seen whether Notch signaling is also involved in ILC1 development, future studies of Notch and ILC biology should take caution in ascribing instructional activities to the pathway when an unbiased, signal integrating model may be a more effective means of understanding how Notch functions within this lineage.

In addition to ILCs, recent studies have also uncovered unappreciated roles for Notch signaling in DC development and function. Though Notch signaling had previously been implicated as an important factor in DC development, where it was thought to be involved in the expansion of pre-DC populations, the extent to which it regulates multiple subsets has only been revealed (Cheng et al., 2003). Studies have now implicated Notch signaling in the development of conventional DCs, splenic CD8⁻ DCs, plasmacytoid DCs (pDC), thymic Thy1⁺ DCs, Esam^{Hi}CD11b⁺ DCs, CD11b⁺CD103⁺ DCs, and even human DC populations (Caton et al., 2007; Ishifune et al., 2011; Lewis et al., 2011; Li et al., 2008; Olivier et al., 2006; Zhou et al., 2009a). Moreover, the Notch pathway is important in regulating cytokine and chemokine production in multiple DC populations following activation (Bugeon et al., 2008; Gentle et al., 2012; Hu et al., 2008; Perez-Cabezas et al., 2011; Svensson et al., 2010). Paralleling its activity in Th cell differentiation, Notch signaling has also been suggested to regulate DC development and function by integrating exogenous stimuli, including TLR ligands and IFN signaling (Gentle et al., 2012; Hu et al., 2008; Svensson et al., 2010). Viewing the role of Notch in DC biology as an integrator of exogenous signals, rather than a lineage instructor, not

only is more consistent with the current literature, but also fits with our understanding of DCs as a dynamic lineage that persists in a wide range of tissues and microenvironments. In this manner, Notch signaling could be understood to allow DCs to efficiently and robustly adapt to a given environment and adopt an appropriate function and fate for a given immune response, even in the face of a complex inflammatory milieu. Further highlighting the similarities in Notch function in Th cell and DC biology, while Notch signaling has been implicated as an important regulator of inflammatory DC function, inhibition of Notch signaling has been found to expand a population of DCs with the capacity to induce Treg differentiation, leading to an accumulation of Tregs *in vivo* (Billiard et al., 2012). Though this is only one report, it is tantalizing to speculate that the Notch pathway functions similarly in both DCs and Th cells in order to coordinate inflammation, at the expense of regulatory responses, thus resulting in the synergistic induction of robust inflammatory responses. Taking this hypothesis further, it would also predict that systemic inhibition of Notch signaling would be particularly efficacious in resolving inflammation and promoting regulatory function, and indeed this phenomena has been observed for models of arthritis, EAE, type-1 diabetes, and GvHD (Billiard et al., 2012; Jiao et al., 2014; Park et al., 2013; Tran et al., 2013; Zhang et al., 2011).

Whether or not Notch plays a non-instructional role in other developmental processes remains to be tested, but recent work on ILCs and DCs illustrates the utility of the signal integration model in interpreting these findings and generating hypothesis for future studies.

Materials and Methods

Mice

ROSA26-DNMAML mice were previously described (Tu et al., 2005). C57Bl/6 mice were obtained from the National Cancer Institute (Frederick, MD). ICN1 transgenic mice were a kind gift from Douglas Melton (Harvard University, MA) and crossed with CD4-Cre (Taconic, NY) or Foxp3-Cre (from Alexander Rudensky, at Memorial Sloan Kettering Cancer Center, NY). *Tbx21*^{-/-} mice were generously provided by John Wherry (University of Pennsylvania, PA). YFP mice, Rag1 deficient mice, SJL mice and C57BL/6 were obtained from Jackson Laboratories (Bar Harbor, ME). All mice were housed in specific pathogen-free facilities at the University of Pennsylvania. Experiments were performed according to the guidelines from the National Institutes of Health with approved protocols from the University of Pennsylvania Animal Care and Use Committee.

TAT-Cre

Expression of TAT-cre was induced with IPTG in bacteria during log phase of growth at 37°C in the presence of chloramphenicol and carbenicillin. The Children's Hospital of Pennsylvania Protein Core carried out purification of TAT-Cre protein. 10⁷ CD4⁺ T cells from DNAML^{FL/FL} or YFP^{FL/FL} mice were MACS purified by positive selection with CD4 Microbeads (Miltenyi). Subsequently, cells were washed, resuspended in 1 ml serum-free OPTI-MEM (Life Technologies), and incubated with 1 ml of 100 mg/ml of TAT-Cre in OPTI-MEM for 12 min at 37°C. Cells were then washed and cultured in IMDM (Life Technologies) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, 50 mM 2-mercaptoethanol, and 100ng/mL IL-7. Twenty-four hours later, naïve YFP⁺ or GFP⁺ CD4⁺ T cells were FACS sorted

Quantitative PCR

RNA was extracted using the QIAGEN RNeasy Mini Kit. cDNA was synthesized from RNA with the Superscript II kit (Invitrogen). Transcripts were amplified with Sybr Green PCR Master Mix (ABI) and quantitative PCR was performed on an ABI ViiA 7 Real-Time PCR System. (Primers, Table S1)

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was performed as described previously (Yashiro-Ohtani et al., 2009). Briefly, chromatin samples were prepared from fixed 6 million cells and immunoprecipitated with rabbit immunoglobulin G (#sc-3888; Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Notch1 antibodies (Aster et al., 2000). Purified DNA was subjected to real-time polymerase chain reaction (PCR) with primers flanking RBPJ binding sites at *Gata3-1a*, *Il4* HS5, *IFNg* CNS-22, or *Tbx21* promoter (primers, Table S2). CD4⁺ T cells from Notch1 null mice were used for an immunoprecipitation negative control. *Nanog* was used as an internal negative control. The DNA quantity recovered from each ChIP sample is shown as the relative value to the DNA input sample.

Luciferase assay

Ifng luciferase constructs are described (Hatton et al., 2006). Constructs included a reporter control (pGL3), the reporter plus a 468 bp *Ifng* minimal promoter (Pro^{WT}), the minimal promoter construct containing a mutated T-box binding site (Pro^{MT}), the minimal promoter construct with a WT *Ifng* CNS-22 upstream (Pro^{WT}CNS^{WT}), the WT CNS-22 construct containing a mutated T-box binding site in the promoter region (Pro^{MT}CNS^{WT}),

the *Ifng* CNS-22 construct with the upstream CNS-22 T-box half-site mutated (Pro^{WT}CNS^{MT1}), and the *Ifng* CNS-22 construct with the downstream CNS-22 T-box half-site mutated (Pro^{WT}CNS^{MT1}). Point mutations to T-box binding sites in the *Ifng* promoter were made using the Promega QuikChange Site Directed Mutagenesis Kit. Constructs were sequenced to prove authenticity. 2×10^5 Jurkat cells were plated in triplicate and transfected with 50 ng of the indicated promoter/reporter constructs, 25 ng pRL-TK, and 100 ng pcDNA-ICN1 or vector control using DMRIE-C (Invitrogen). Transfected cells were cultured overnight and then stimulated for 4 hours with 20 ng/mL PMA and 200 ng/mL ionomycin. 2×10^4 U2OS cells were plated in triplicate and transfected with 50 ng of the indicated promoter/reporter constructs, 10 ng pRL-TK, 10 ng pcDNA-ICN1 or vector control, and 25 ng Mig-Tbet or vector control using FuGENE 6 (Promega). After stimulation, firefly substrate activity was measured using Britelite Plus (PerkinElmer) and renilla substrate activity measured using Stop & Glo (Promega). Firefly values were normalized to Renilla and then all normalized values set relative to the pGL3 control vector. All measurements were performed using a GloMax-96 Microplate Luminometer (Promega).

***In vitro* T cell culture**

Lymph nodes from WT mice were CD4⁺ MACS purified and then naïve CD4⁺ T cells were FACS sorted. Naïve CD4⁺ T cells were co-cultured with irradiated, Thy1.2 depleted splenocytes at a 1:5 ratio and stimulated using anti-CD3e (1 µg/mL), and anti-CD28 (1 µg/mL) mAb. Cells were cultured under either neutral (5 ng/mL IL-2, 20 µg/mL anti-IL-4, and 20 µg/mL anti-IFNγ), Th1 (5 ng/mL IL-2, 5 ng/mL IL-12, and 20 µg/mL anti-IL-4), Th2 (20 ng/mL IL-4, 20 µg/mL anti-IFNγ, and 20 µg/mL anti-IL-12), Th9 (10 ng/mL IL-4 and 5ng/mL TGF-β), Th17 (20 ng/mL IL-6 and 5ng/mL TGF-β), or Treg (5 ng/mL IL-2

and 5ng/mL TGF- β) culture conditions. For titration experiments, the conditions listed above were used for 1x dilutions and all other dilutions were made from this stock. For GSI experiments, cells were treated with either DMSO or 1 μ M GSI.

Adoptive T cell transfer

Total T cells and non-T cells from spleen and lymph nodes of CD4Cre-YFP mice or CD4Cre-ICN1 mice were purified and sorted with thy1.2 Abs. CD4 T cells and CD8 T cells from spleen and lymph nodes of CD4Cre-YFP mice or CD4Cre-ICN1 mice were positively selected with thy1.2 (30-H12) then sorted with anti-CD4 (RM4-5) and anti-CD8a (53-6.7) 2×10^6 cells were transferred to B6 mice and frequency of Gr1+(RP6-8C5) and CD11b+ (M1/70) population in peripheral blood were analyzed after 4 weeks of transfer.

Mixed bone marrow chimeras

5FU treated BM cells (BMCs) from WT (SJL) and CD4Cre-ICN1 mice were adoptively transferred into sub-lethally irradiated (500 rad) Rag1 deficient mice with 1:1 mixture of BMCs from WT (CD45.1; 2×10^6 BMCs) and/or CD4Cre-ICN1 (CD45.2; 2×10^6 BMCs) mice. Recipient Rag1 deficient mice were treated with acidified, antibiotic water 1 week before to 2 weeks after the irradiation and then switched to acidic water without antibiotics for 4 weeks. Mice were analyzed 6 weeks after the transfer.

Measurement of cytokines

For intracellular cytokine staining, cells were restimulated, fixed, and stained as described (Tu et al., 2005). Cells were stained with anti-CD4 (RM4-5, Biolegend), anti-IFN γ (XMG1.2, BD), anti-IL4 (11B11, eBioscience), anti-IL17A (ebio17B7, eBioscience),

anti-Tbet (eBio4B10, eBioscience), and anti-GATA3 (L50-823, BD). Cells were acquired on a LSRII (Becton Dickinson) and data was analyzed with FlowJo (TreeStar). ELISA was performed as described (Tu et al., 2005).

GSI-washout assay

WT CD4⁺ T cells were stimulated as above and 24 hours post-stimulation cells were treated with either DMSO or GSI (1 μ M) for 20 hours. Subsequently, T cells were CD4⁺ MACS purified and cells cultured in media containing cycloheximide (20 μ g/mL) and cells previously cultured DMSO were replated in DMSO and cells cultured in GSI were either replated in 1 μ M GSI or in DMSO for 4 hours. RNA was then harvested and analyzed by qPCR.

***T. muris* infection and antigen**

T. muris eggs were prepared as described (Tu et al., 2005). Mice were infected on day 0 with 150–200 embryonated eggs, and parasite burdens were assessed on day 21 after infection. Mesenteric LN (MLN) cell suspensions were prepared and resuspended in IMDM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 g/ml streptomycin, 2 mM L-glutamine, and 50 M 2-mercaptoethanol. Cells were plated at 4 X 10⁶ cells/well and cultured in the presence of anti-CD3e and anti-CD28 for 72 hours. Levels of IL-4, -5, and -13 were assayed by sandwich ELISA. For histology, segments of mid-cecum were removed, washed in sterile PBS, and fixed for 24 hours in paraformaldehyde. Tissues were processed and paraffin embedded using standard histological techniques. For detection of intestinal goblet cells, 5 μ m sections were cut and stained with hematoxylin and eosin or Alcian blue periodic acid Schiff. Analysis of parasite- specific IgG1 and IgG2c production was performed by antigen capture.

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